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LARISSA TUANNY FRANCO

Exposure Assessment to Multiple Mycotoxins in Rural Areas of São Paulo and Santa Catarina States, Brazil

Pirassununga

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Tese apresentada à Faculdade de Zootecnia e Engenharia de Alimentos da Universidade de São Paulo, como parte dos requisitos para obtenção do título de Doutora em Ciências.

Área de concentração: Qualidade e Produtividade Animal

Orientador: Dr. Carlos Augusto Fernandes de Oliveira

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"Aos outros, dou o direito de ser como são. A mim, dou o dever de ser cada dia melhor.
[Chico Xavier]

"What we think, or what we know, or what we believe is, in the end, of little consequence. The only consequence is what we do."
[John Ruskin]

ABSTRACT

FRANCO, L.T. **Exposure assessment to multiple mycotoxins in rural areas of São Paulo and Santa Catarina States, Brazil.** 2019. 195 p. PhD Science Dissertation School of Animal Science and Food Engineering, University of São Paulo, Pirassununga.

Mycotoxins are secondary metabolites produced by fungi that occur naturally in foodstuffs, which can cause a large variety of toxic effects on vertebrates including humans. The objectives of this work were to evaluate the co-occurrence of 11 mycotoxins in food products, feed for broiler chicks, laying hens and dairy cattle, assess the human exposure to mycotoxins through food analysis versus consumption data and multi-mycotoxin biomarkers in urine, and characterize the associated risk of mycotoxin exposure in Brazilian rural areas. Sampling procedures were conducted in 38 small-scale dairy and poultry farms in the surroundings of Pirassununga and Descalvado (State of São Paulo), Pinhalzinho and Erval Velho (State of Santa Catarina). In these farms a total of 86 volunteers were recruited and instructed to provide samples of the morning urine (N = 162) in two sampling periods (April-May/2016 and December/2016), along with samples of rice (N = 66), bean (N = 59), wheat (N = 39), corn flour (N = 21) and corn meal (N = 18) available in their households. Samples of feed for broilers (N = 10), laying hens (N = 20) and dairy cattle (N = 15) were also collected. All samples were analyzed by ultra-performance liquid chromatography coupled to mass spectrometry (UPLC-MS/MS) for determination of aflatoxins (AF) B_1, B_2, G_1 and G_2, fumonisins (F) B_1 and B_2, ochratoxin A (OTA), zearalenone (ZEN), deoxynivalenol (DON), toxin T-2 and toxin HT-2 in food products and feeds, and AFM_1, AFP_1, AFQ_1, FB_1, OTA, T-2, HT-2, DON, de-epoxideoxynivalenol (DOM-1), ZEN, α-zearalenol (α-ZEL), β-zearalenol (β-ZEL) and 15-acetyl-DON in urine samples. The mycotoxin levels in urine were adjusted to creatinine concentration in each sample analyzed. In feed samples, median levels of total AF, total FB, ZEN and DON were 100 μg/kg, 680 μg/kg, 160 μg/kg and 200 μg/kg, respectively. The co-occurrence of two or more mycotoxins was confirmed in 51% of feed samples. Results indicate a high exposure of farm animals to mycotoxins in the feed, hence emphasizing the need to improve the feed quality regarding the contamination with mycotoxins in small-scale farms in Brazil, and the necessity of include feed in Brazilian regulation, especially for AF, FB, and ZEN. Mycotoxin levels above the Brazilian maximum permitted levels (MPL) were found in rice (1.5%), wheat flour (12.8%) and corn flour (14.3%) samples. Urine determinations revealed the presence of AFM_1 and AFP_1, DON, OTA, FB_1 and ZEN at levels of 0.02-12.0 ng/mg creatinine. Regarding the probable daily intake (PDI) based on food data, only ZEN (0.156 μg/kg b.w./day) had a Hazard Quotient (HQ) above the tolerance (> 1). PDI values based on urinary levels for DON, OTA and total AF were 84.914, 0.031 and 0.001 μg/kg b.w./day, respectively, resulting in HQ values > 1, which may indicate health risks for the population studied. An informal intervention by means of educational activities and delivery of an information flyer during the first sampling period did not change the exposure levels to mycotoxins in the second sampling period. Further studies are needed to identify food items other than those analyzed in this work as sources of dietary mycotoxins, as well as the contribution of inhalation of contaminated dusts for the exposure. This is the first study to report the risk assessment of mycotoxins based on food and urinary levels in rural areas in Brazil.

Keywords: Mycotoxins; Food; Feed; Urine; Biomarker; Analysis; Risk assessment.

RESUMO

FRANCO, L.T. **Avaliação da exposição a múltiplas micotoxinas em áreas rurais dos estados de São Paulo e Santa Catarina, Brasil.** 2019. 195 f. Tese (Doutorado) – Faculdade de Zootecnia e Engenharia de Alimentos, Universidade de São Paulo, Pirassununga.

As micotoxinas são metabólitos secundários produzidos por fungos que ocorrem naturalmente em alimentos, das quais podem causar uma grande variedade de efeitos tóxicos em vertebrados, incluindo humanos. Os objetivos deste trabalho foram avaliar a co-ocorrência de 11 micotoxinas em alimentos, rações para frangos de corte, poedeiras e gado leiteiro, avaliar a exposição humana a micotoxinas através de análise de alimentos versus dados de consumo e biomarcadores de múltiplas micotoxinas na urina, e caracterizar o risco associado de exposição a micotoxinas em áreas rurais brasileiras. Os procedimentos de amostragem foram conduzidos em 38 propriedades leiteiras e avícolas de pequeno porte nos arredores de Pirassununga e Descalvado (SP), Pinhalzinho e Erval Velho (SC). Nestas fazendas, um total de 86 voluntários foram recrutados e instruídos a fornecer amostras da primeira urina da manhã (N = 162) em dois períodos de amostragem (abril-maio/2016 e dezembro/2016), juntamente com amostras de arroz (N = 66) , feijão (N = 59), trigo (N = 39), farinha de milho (N = 21) e fubá (N = 18) disponíveis em suas residências. Amostras de ração para frangos de corte (N = 10), poedeiras (N = 20) e bovinos leiteiros (N = 15) também foram coletadas. Todas as amostras foram analisadas por cromatografia líquida de ultra-performance acoplada a espectrometria de massas (UPLC-MS/MS) para determinação de aflatoxinas (AF) B_1, B_2, G_1 e G_2, fumonisinas (FB) B_1 e B_2, ocratoxina A (OTA), zearalenona (ZEN), desoxinivalenol (DON), toxina T-2 e toxina HT-2 em produtos alimentícios e rações, e AFM_1, AFP_1, AFQ_1, FB_1, OTA, T-2, HT-2, DON, de-epóxido-oxinivalenol (DOM-1), ZEN, α-zearalenol (α-ZEL), β-zearalenol (β-ZEL) e 15-acetil-DON em amostras de urina. Os níveis de micotoxinas na urina foram ajustados à concentração de creatinina em cada amostra analisada. Em amostras de ração, os níveis médios de AF total, FB total, ZEN e DON foram de 100 µg/kg, 680 µg/kg, 160 µg/kg e 200 µg/kg, respectivamente. A co-ocorrência de duas ou mais micotoxinas foi confirmada em 51% das amostras de ração. Os resultados indicam uma alta exposição de animais de fazenda à micotoxinas na ração, enfatizando a necessidade de melhorar a qualidade das rações em fazendas de pequena escala no Brasil, referente as micotoxinas, e a necessidade de incluir ração na legislação brasileira, especialmente para AF, FB e ZEN. Os níveis de micotoxinas acima dos níveis máximos permitidos no Brasil (LMP) foram encontrados em arroz (1,5%), farinha de trigo (12,8%) e farinha de milho (14,3%). As determinações da urina revelaram a presença de AFM_1 e AFP_1, DON, OTA, FB_1 e ZEN nos níveis de 0,02-12,0 ng/mg de creatinina. Em relação à ingestão provável diária (IPD) com base em dados de alimentos, apenas ZEN (0,156 µg/kg p.c./dia) apresentou um Quociente de Risco (HQ) acima do tolerável (> 1). Os valores de IPD baseados nos níveis urinários para DON, OTA e AF total foram 84,914, 0,031 e 0,001 µg/kg p.c./dia, respectivamente, resultando em valores de HQ> 1, o que pode indicar riscos para a saúde da população estudada. Uma intervenção informal por meio de atividades educacionais e entrega de um folheto informativo durante o primeiro período de amostragem não alterou os níveis de exposição às micotoxinas no segundo período de amostragem. Mais estudos são necessários para identificar itens alimentares além dos analisados neste trabalho como fontes de micotoxinas diárias, bem como a contribuição da inalação de pós contaminados para a exposição. Este é o primeiro estudo a relatar a avaliação de risco de micotoxinas com base em alimentos e níveis urinários em áreas rurais no Brasil.

Palavras-Chave: Micotoxinas. Alimentos. Rações. Urina. Biomarcador. Análise. Avaliação de risco.

LIST OF ABBREVIATIONS, SYMBOLS AND ACRONYMS

µg	Microgram
µL	Microliter
pg	Picogram
AF	Aflatoxin
AFB_1	Aflatoxin B_1
AFB_1-N^7-gua	Aflatoxin B_1-N^7-guanine
AFB_2	Aflatoxin B_2
AFG_1	Aflatoxin G_1
AFG_2	Aflatoxin G_2
AFL	Aflatoxicol
AFM_1	Aflatoxin M_1
AFP_1	Aflatoxin P_1
AFQ_1	Aflatoxin Q_1
CHC	Hepatocellular carcinoma
CYP	Cytochrome P450
DOM-1	De-epoxydeoxynivalenol
DON	Deoxynivalenol
FB_1	Fumonisin B_1
FB_2	Fumonisin B_2
g	Gramme
HT-2	Toxin HT-2
IARC	International Agency for Research on Cancer
kg	Kilogram
µg/kg b.w./day	Microgram/kilogram of body weight/day
LC-MS/MS	Liquid chromatography coupled to mass spectrometry
LOD	Detection limit
LIQ	Lower Limit of Quantification
LOQ	Limit of Quantification
mg	Miligram
min	Minute
ml	Mililiter
mm	Milímeter
MRM	Multiple reaction monitoring
NIV	Nivalenol
nm	Nanmeter
OTA	Ochratoxin A

rpm	Rotations per minute
T-2	Toxin T-2
UHT	Ultra-High temperature
ZEN	Zearalenone
α-ZEL	α-Zearalenol
β-ZEL	β-Zearalenol
MPL	Maximum permitted level adopted
PDI	Probable Daily Intake
TDI	Tolerable Daily Intake
HQ	Hazard Quotient
MoE	Margin of Exposure
ANVISA	*Agência Nacional de Vigilância Sanitária* (National Sanitary Surveillance Agency)
EFSA	European Food Safety Authority
FAO	Food and Agriculture Organization of United Nations
WHO	World Health Organization

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SUMMARY

1 Introduction

Mycotoxins are organic compounds of low molecular weight and diverse chemical structure produced as secondary metabolites by various species of fungi during growth on agricultural products, especially in cereals (ABBAS, 2005). Diseases in humans and animals caused by mycotoxins are called mycotoxicoses, which are diffuse syndromes characterized by lesions in target organs such as the liver, kidneys, epithelial tissue (skin and mucous membranes) and central nervous system, depending on the type of toxin (DIAZ, 2005). The major groups of toxigenic fungi and their respective mycotoxins are distributed in three genuses as follows (OLIVEIRA; CORRÊA, 2010):

a) *Aspergillus* genus, mainly *A. flavus*, *A. parasiticus* and *A. nomius*, which produce the aflatoxins (AF);
b) *Fusarium* genus, the main mycotoxins produced are fumonisins (FB), trichothecenes including deoxynivalenol (DON) and zearalenone (ZEN);
c) *Aspergillus alutaceus* (formerly known as *A. ochraceus*) and species of the genus *Penicillium*, which produce ochratoxin A (OTA).

The mycotoxin contamination of food leads to economic losses and is considered as a serious public health issue, since the dietary exposure may originate a variety of toxic effects in humans, ranging from acute toxicity to chronic diseases including cancer (WILD; GONG, 2009). In addition, the co-occurrence of mycotoxins in foods is also possible, which increases the health risk as result of consumption of these products due to the possibility of interaction of toxic effects caused by different mycotoxins (CAST, 2003). In animal models, the toxicity of some individual mycotoxins can be increased synergistically, additive or antagonistic when they occur as co-contaminants and are consumed by different species (OLIVEIRA; CORRÊA, 2010). In Brazil, the occurrence of mycotoxins in food has been well documented in several studies, indicating high frequencies and concentrations of AF, FB and, more recently, DON, especially in corn, peanuts, wheat, milk and derived products (ABIA et al., 2013; AFSHAR et al., 2013; ALKADRI et al., 2014; ASSEM; MOHAMAD; OULA, 2011; BENSASSI et al., 2010; BORDIN et al., 2015; CALORI-DOMINGUES et al., 2016; CANO-SANCHO et al., 2012; DE BOEVRE et al., 2012, 2013; DING et al., 2012; GONZÁLEZ-OSNAYA et al., 2011; JAKŠIĆ et al., 2012; JI et al., 2014; LEWIS et al., 2005; LI et al., 2014; LINDBLAD et al., 2013; MARTINS et al., 2012a; NJOBEH et al., 2010; RIZZO; ESKOLA; ATROSHI, 2002; RODRÍGUEZ-CARRASCO et al., 2012; SANTOS et al., 2010; SERRANO et al., 2012; SETYABUDI et al., 2012; SULYOK; KRSKA; SCHUHMACHER, 2010; STANCIU et al., 2017; XU; HAN; LI, 2019; ZEBIRI et al.,

2018). Although the published data indicate a high potential for consumption of mycotoxins individually in contaminated foods by the population, there are no studies in Brazil aiming to evaluate the simultaneous exposure to different mycotoxins in the diet.

The exposure to dietary mycotoxins has been traditionally assessed on the basis of their intake from food (or feed), also known as “external exposure” or “oral dose”, which is calculated considering the mycotoxin occurrence in food products and the consumption data, leading to a probable daily intake (PDI) value (OLIVEIRA, 2016). Because of limitations inherent to that approach, especially for the individual measurement of those variables, biomarkers have been proposed as a suitable alternative whereby a more accurate assessment of exposure at the individual level can be performed (OLIVEIRA, 2016). Biomarkers measure a cellular, biological or molecular change in a biological environment (human tissues, cells or fluids) that provides information about a disease or exposure to a particular substance (JAGER et al., 2011). Biomarkers are used to measure or indicate a biological process, and therefore the detection of specific biomarkers may aid in the identification, diagnosis and treatment of affected individuals who may be at risk but still do not exhibit the symptoms (JAGER et al., 2011). The development of biomarkers for environmental agents should be based on specific knowledge of metabolism, product formation and general mechanism of action (GROOPMAN; KENSLER, 1999).

Biomarkers can be used to indicate four sequential events after the ingestion of a toxic compound: internal dose, biologically effective dose, early biological effect, and structural/function changes or disease. The internal dose is the amount of substance that is internalized in the body and metabolized or excreted (OLIVEIRA, 2016). In this context, biomarkers-based approaches to assessing human exposure to mycotoxins have gained wider acceptance, since specific biomarkers present in the urine are related to mycotoxin ingestion (WARTH; SULYOK; KRSKA, 2013). Urinary biomarkers suitable for AFB_1 and ZEN are AFM_1, AFP_1 and AFQ_1 (GROOPMAN; KENSLER, 1999), and non-metabolized ZEN + α-zearalenol (α-ZEL) + β-zearalenol (β-ZEL) (SOLFRIZZO; GAMBACORTA; VISCONTI, 2014), respectively (SOLFRIZZO; GAMBACORTA; VISCONTI, 2014). Non-metabolized FB_1, OTA and DON + de-epoxideoxynivalenol 1 (DOM-1) + 15-acetyl-DON (15-Ac-DON) are urinary biomarkers for FB_1, OTA and DON, respectively (SOLFRIZZO; GAMBACORTA; VISCONTI, 2014). Some of these biomarkers are excreted in free and conjugated forms, which requires the use of the enzyme β-glucuronidase / sulfatase in urine sample to release the conjugated forms and

increase the concentration and detectability of the free analytes (SOLFRIZZO et al., 2011).

In recent years, multi-analyte methods based on liquid chromatography tandem mass spectrometry (LC-MS/MS) has been successfully introduced into the field of mycotoxin analysis, opening new perspectives for the evaluation of co-occurring mycotoxins in food products and several biomarkers in a single sample (HEYNDRICKX et al., 2015; HUYBRECHTS et al., 2015; LI et al., 2018; SOLFRIZZO; GAMBACORTA; VISCONTI, 2014; WARTH; SULYOK; KRSKA, 2013) Several pilot scale studies that apply multi-biomarker approaches to investigate the mycotoxin exposure of populations in Africa, Asia, or Europe have revealed regional differences in urinary biomarkers excretion patterns (GERDING et al., 2015; SONG et al., 2013; WARTH et al., 2014). However, so far, no study attempted to measure the exposure to dietary mycotoxin using urinary multi-biomarker approaches in Brazil. In fact, only three single biomarker studies were performed to assess the AF exposure in the state of São Paulo (JAGER et al., 2014, 2016; ROMERO et al., 2010). Moreover, Bordin et al. (2015) evaluated the FB exposure in individuals from two cities from the state of São Paulo (Pirassununga and Descalvado) and rural areas in the surroundings of Pinhalzinho and Erval Velho from Santa Catarina state using the mycotoxin occurrence levels in food products and the consumption data, as well as the levels of FB_1 in samples of human hair. The outcomes from those studies indicated significant human exposure in the two Brazilian states mentioned to dietary AF and FB. Thus, the possibility of additional exposure to other mycotoxins through the food in those areas seems logical and requires proper evaluation to assess the associated health risks.

Because of its high continental dimension, Brazil has considerable climatic heterogeneity, with several types of soil and topography (ASSAD et al., 2004). For example, the before mentioned cities of Pirassununga and Descalvado are located in the Center-East region of the state of São Paulo, which has a predominant tropical climate of altitude and rainy season from October to March (SANTOS; MINCATO, 2005). Santa Catarina is one of the Brazilian states that presents a better distribution of rainfall during the year, having of subtropical climate and lower precipitation in the fall, and increased heat in the summer associated with high moisture indexes which favors the formation of tropical convection (MONTEIRO, 2001). Both cities in the states of São Paulo (Pirassununga and Descalvado) and Santa Catarina (Pinhalzinho and Erval Velho) have extensive agricultural activities based on small-scale farms, especially for production of milk, egg and broiler. Pirassununga has a population of 70,138 inhabitants (57,594 in the

urban area and 7,270 in the rural area) with an area of 727 km² and altitude of 627 meters (PREFEITURA DE PIRASSUNUNGA, 2018). The estimated population for Descalvado is 31,056 inhabitants (24,136 in the urban area and 4,785 in the rural area), with a 755,226 km² at 679 meters of altitude (PREFEITURA DE DESCALVADO, 2018). Pinhalzinho has a population of 19,511 inhabitants, with a 128.298 km² at 660 meters of altitude. The agricultural economy accounts for 37% of the municipality's revenue with 850 farms mainly based on milk and corn, and swine and poultry production (PREFEITURA DE PINHALZINHO, 2018). The Municipality of Erval Velho has 4,456 inhabitants with a 207.4km² at 674 meters of altitude. The municipality's economy is based on farming, the recycling industries and family trade. It is marked by influences from German and Italian culture, including eating habits such as the consumption of cereals, pasta and polenta. The agricultural economy stands out by the production of milk, swine, poultry, as well as soybean, corn and bean cultivation for family subsistence (PREFEITURA DE ERVAL VELHO, 2018).

In summary, the aforementioned aspects clearly indicate that there is little information in Brazil on the human exposure to mycotoxins based on urinary biomarkers, except for the reports from Romero et al. (2010) and Jager et al. (2016), and that there is no study evaluating the simultaneous exposure to several mycotoxins possibly present in the human diet or animal feed. Therefore, the present study was designed to evaluate the human exposure to co-occurring mycotoxins in food products through the mycotoxin occurrence levels versus consumption data and by multi-mycotoxin biomarkers in human urine aiming to characterize the associated risks, as well as to assess the mycotoxin co-occurrence in the animal feed in rural areas of São Paulo and Santa Catarina, Brazil.

1.1 General Objective

The objectives of this work were to evaluate the co-occurrence of 11 mycotoxins in food products, feeds for chicken broiler chicks, laying hens and dairy cattle, assess the human exposure to mycotoxins through multi-mycotoxin biomarkers in urine, and thereby characterize the associated risk of mycotoxin exposure in Brazilian rural areas. For these purposes, LC-MS/MS analytical methods were validated and applied for determination of major mycotoxins in food and feed products, as well as multi-mycotoxin biomarkers in urine from residents in small-scale rural properties in the states of São Paulo and Santa Catarina, Brazil. The effect of simple intervention activities focused on education about grain and feed storage practices was also evaluated. Finally, a risk characterization was performed to assess whether there are public health concerns

regarding mycotoxin exposure of the population studied based on the direct measurement of daily dietary intake and the estimation of exposure from urinary biomarkers of mycotoxins.

1.2 Literature Review

1.2.1 Occurrence of Mycotoxins in Food Products

Humans and animal have been threatened by mycotoxins in foodstuffs since ancient times. It is generally assumed that some historical facts would be related to the ingestion of mycotoxins, as for example the last one of the Ten Pests of Egypt, which could be a description of cases of ergotism caused by the consumption of grains contaminated with *Claviceps* fungi (MILLER, 1995). In the Middle Ages, ergotism was re-named as the "Fire of St. Anthony" (RICHARD, 2007). Another example was the episode known as "The Witches of Salem" in the year 1692 in Massachusetts, United States, which is attributed to failed crops combined with climatic events favorable to *Claviceps* proliferation that forced the use of contaminated rye to produce bread (RICHARD, 2007). However, only after the discovery of AF in the early 1960's the toxigenic fungi and their respective mycotoxins started to attract great attention from scientists, leading to intensive research on the occurrence of mycotoxins in food products (OLIVEIRA et al., 2014).

Considering the toxicological properties of mycotoxins, several countries established limits of tolerance for these compounds in foodstuffs. In Brazil, the Resolution RDC No. 07 of February 18, 2011 established maximum permitted levels (MPL) for total AF (sum of AFB_1, AFB_2, AFG_1 and AFG_2), DON, OTA, total FB (sum of FB_1 and FB_2), and ZEN in the most susceptible foods for contamination, including cereals and cereal-based products, such as rice, bean, wheat flour, corn flour, and corn meal, among others (ANVISA, 2011). Despite regulations, the occurrence of mycotoxins in Brazilian food products has been highlighted by several studies conducted in the past 10 years (Table 1), indicating high frequencies and concentrations of total AF, total FB and, more recently, DON, especially in corn, peanuts, wheat, and products made with these cereals. When comparing the results reported with the MPL adopted by Brazilian regulations (ANVISA, 2011), it is possible to observe that corn is one of the most frequent foods with levels of FB and OTA above the regulated values (MPL of total FB and OTA in corn: 1500 μg/kg and 20 μg/kg, respectively).

Table 1 - Occurrence of the main mycotoxins in food in the last 10 years in Brazil

continue

Type of food	Mycotoxin	Mycotoxin levels		n / N (%)	Reference
		Range (µg/kg)	Mean ± SD (µg/kg)		
Raw peanut	AFB_1+AFB_2+AFG_1+AFG_2	0.5 – 103.8	12.88 ± 2.42	106/240 (44)	(OLIVEIRA et al., 2009)
Peanut candy			8.97 ± 1.61		
Salted peanut			1.60 ± 0.25		
Salted and coated peanuts			3.32 ± 0.67		
Sweet peanut			3.32 ± 0.67		
Corn	AFB_1+AFB_2+AFG_1+AFG_2	5 - 54	24.1	24/300 (8)	(MORENO et al., 2009)
	FB_1+ FB_2	10 – 18,160	2,540	179/180 (99)	
Corn	FB_1 + FB_2	15 – 9,670	1,115	86/149 (58)	(ROCHA et al., 2009)
Rice	AFB_1+AFB_2+AFG_1+AFG_2	ND - 176.31	9.37 ± 1.86	135/230 (59)	(ALMEIDA et al., 2012)
	OTA	ND - 30.24	1.78 ± 0.64	92/230 (40)	
	ZEN	ND – 4,872.5	143.0 ± 26.4	104/230 (45)	
	DON	ND - 244.00	119.33 ± 11.6	19/230 (8)	
Corn meal	FB_1 + FB_2	45 – 2,322	326	28/29 (97)	(MARTINS et al., 2012a)
Corn grits		45 – 3,462	292.5	19/29 (65)	
Popcorn		57 – 1,170	223	15/17 (88)	
Corn flour		56 – 555	170	11/15 (73)	
Corn flakes		50 - 840	214	9/11 (82)	
Wheat grain	DON	ND – 1,250	540	65/65 (100)	(DEL PONTE; GARDA-BUFFON; BADIALE-FURLONG, 2012)
	NIV	ND - 781	337	54/65 (83)	

Table 1 - Occurrence of the main mycotoxins in food in the last 10 years in Brazil

conclusion

Type of food	Mycotoxin	Mycotoxin levels		n / N (%)	Reference
		Range (µg/kg)	Mean ± SD (µg/kg)		
Corn products	FB_1+ FB_2 + FB_3	230 – 6,450	NR	40/40 (100)	(QUEIROZ et al., 2012)
	ZEN	1.8 - 99	NR	38/40 (95)	
Peanut	AFB_1+AFB_2+AFG_1+AFG_2	ND - 36.7	8.5 ± 12.7	8/23 (35)	(JAGER et al., 2013)
Corn derivatives	AFB_1+AFB_2+AFG_1+AFG_2	ND - 8.3	NR	29/80 (36)	
Bean	AFB_1+AFB_2+AFG_1+AFG_2	ND - 0.042	0.10 ± 0.09	38/51 (75)	
Fluid milk	AFM_1	ND - 0.069	0.03 ± 0.02	26/65 (40)	
Powdered milk	AFM_1	ND - 0.81	0.65 ± 0.22	2/4 (50)	
Cheese	AFM_1	ND - 0.030	0.16 ± 0.12	3/10 (30)	
Wheat	DON	206.3 – 4,732.3	1894.9	75/113 (66)	(SANTOS et al., 2013)
Corn meal	FB_1	NR	422.9 ± 139.0	13/18 (72)	(BORDIN et al., 2015)
Cornflakes		NR	170.8 ± 43.5	3/6 (50)	
Popcorn		NR	537.4 ± 922.2	11/15 (73	
Canned corn		ND	ND	0/4 (0)	
Polenta		NR	214.2 ± 89.5	3/7 (43)	

n / N: Number of positive samples / number of samples analyzed; ND: not detected; NR: not reported. UHT: Ultra-High Temperature. AFB_1: aflatoxin B1; AFB_2: aflatoxin B_2; AFG_1: aflatoxin G1; AFG_2: aflatoxin G2; AFM_1: aflatoxin M1; FB_1: fumonisin B1; FB_2: fumonisin B2; FB_3: fumonisin B3; ZEN: zearalenone; DON: deoxynivalenol; NIV: nivalenol; OTA: ochratoxin A.

Although the mean levels of ZEN in rice were within the Brazilian MPL (150 µg/kg), Almeida et al. (2012) found levels up to 4,872 µg/kg, which is much higher than the tolerable level for this mycotoxin. Additionally, wheat samples contained high levels of DON (SANTOS et al., 2013), and rice and peanut samples also showed levels higher than the MPL for total AF and DON (ALMEIDA et al., 2012; OLIVEIRA et al., 2009). Among the data presented in Table 1, only beans and milk samples were in accordance with the Brazilian MPL for total AF and AFM_1, respectively. Therefore, the data presented in Table 1 indicate a high intake of mycotoxins by the Brazilian population through the consumption of food products containing levels above the MPL, hence emphasizing the importance of studies on the assessment of human exposure in Brazil.

1.2.2 Occurrence of Mycotoxins in Feed

In 1960, nearly one hundred thousand turkeys died in England due to the consumption of ration produced with peanut meal imported from Brazil. After the successful reproduction of the disease by administering the extract of *A. flavus* cultures, and the proof of the involvement of its fungal metabolite, the disease was called "Turkey X disease" (SARGEANT et al., 1961). Currently, the occurrence of mycotoxins in feed continues as the one of major causes of economic loss in animal production, leading to general negative effects such as decreased body weight, food refusal, immunosuppression, worst feed conversion, decrease in egg production and increase in mortality (RICHARD, 2007).

Considering the risks posed by mycotoxins to animal raising activities, tolerance limits for these toxins have been adopted for ingredients and animal feed in many countries (OLIVEIRA et al., 2014). However, in Brazil there is no regulation for the levels of mycotoxins in feed, except for a recommend level of 50 µg/kg for total AF in feed ingredients (BRASIL, 1988). The occurrence of mycotoxins in corn is of particular great concern, because it is the main ingredient (~60%) used in feed for poultry, swine and dairy cows in Brazil and other countries (MARTINS et al., 2012b; OLIVEIRA et al., 2006). Fungi are abundant in the environment and therefore all feedstuffs can be contaminated with mycotoxins, since they are not restricted to any element of the animal feed supply chain. Moreover, the level of contamination may vary according to the geographical location, agronomic practices and climatic conditions, which overall influence the type of fungi that are prevalent in a farming system (BRYDEN, 2012).

There are a relatively low number of field studies reporting the occurrence levels of mycotoxins in feed samples in Brazil in the past 10 years, as shown in Table 2. Most

studies reported the incidence of AF or FB, alone or in combination. AF levels higher than the Brazilian recommend level of 50 µg/kg (BRASIL, 1988) were observed for all types of feed analyzed, at concentrations ranging from 1.4 to 194.51 µg/kg. The incidences of AFB_1 in feed for dairy cows in two studies available were 31% (MOTTA et al., 2015) and 40% (OLIVEIRA, 2010) of samples analyzed, although the levels were much higher (1.68-194.51 µg/kg) in the first study (MOTTA et al., 2015). FB levels of up to 1,810 µg/kg in broiler feed were reported by Rossi et al. (2013) in 91-100% of samples analyzed, although the levels were below to guidance levels for complete feedingstuffs for poultry adopted in Europe (EUROPEAN COMMISSION, 2006).

In the only study available with feed for pigs in Brazil in the past 10 years, OTA was detected in 31% of samples analyzed at levels of 36 - 120 µg/kg (ROSA et al., 2009). Souza et al. (2013) studied for the first time in Brazil the co-occurrence of several mycotoxins in corn and in poultry feed and confirmed the high incidence of FB (100% of positive samples) at levels of 58-1,592 µg/kg, along with relative low concentrations of AFG_2, DON, ZEN, nivalenol, and hydrolised FB_1. The authors observed that the mean levels of FB in poultry feed were lower than in corn samples, which was attributed to possible degradation of the mycotoxin during feed manufacture, or a dilution effect after adding other ingredients besides corn. These studies reinforce the need for further investigations on the occurrence of mycotoxin mixtures in feed in Brazil.

Table 2 - Occurrence of the main mycotoxins in animal feed in the last 10 years in Brazil

Animal	Mycotoxin	Mycotoxin levels		n / N (%)	Reference
		Range (µg/kg)	Mean (µg/kg)		
Swine	OTA	36 - 120	NR	8/26 (31)	(ROSA et al., 2009)
Dairy cow	AFB_1	1 – 19.5	NR	12/30 (40)	OLIVEIRA (2010)
Broiler	AFB_1	0.79 – 60.80	8.41	30/34 (88)	(ROSSI et al., 2012)
Laying hens	AFB_1	1.03 – 91.04	19.75	33/36 (92)	
Broiler (pre-starter)	FB_1+FB_2	310 – 1,000	680	18/18 (100)	(ROSSI et al., 2013)
	$AFB_1+AFB_2+AFG_1+AFG_2$	1.4 – 3.5	2.2	9/18 (50)	
Broiler (starter)	FB_1+FB_2	190 – 2,150	600	33/33 (100)	
	$AFB_1+AFB_2+AFG_1+AFG_2$	2.2 – 46.3	6.1	16/33 (48)	
Broiler (grower)	FB_1+FB_2	100 – 1,810	680	68/74 (92)	
	$AFB_1+AFB_2+AFG_1+AFG_2$	1.9 – 60.4	6.4	60/74 (81)	
Broiler (finisher)	FB_1+FB_2	120 – 1,190	520	30/33 (91)	
	$AFB_1+AFB_2+AFG_1+AFG_2$	2 – 5.6	3.6	29/33 (88)	
Poultry	FB_1+FB_2	58 – 1,592	NR	36/36 (100)	(SOUZA et al., 2013)
	AFB_1	ND	ND	0	
	AFB_2	ND	ND	0	
	AFG_1	ND	ND	0	
	AFG_2	ND – 1.43	NR	5/36 (14)	
	DON	ND – 20	NR	2/36 (3)	
	NIV	ND – 67	NR	6/36 (17)	
	ZEN	ND – 6.5	NR	14/36 (39)	
	HFB_1	ND	ND	0	
Dairy cow	AFB_1	1.68 – 194.51	15.69	27/89 (31)	(MOTTA et al., 2015)

n / N: Number of positive samples / number of samples analyzed; ND: not detected. NR: not reported. AFB_1: aflatoxin B_1; AFB_2: aflatoxin B2; AFG_1: aflatoxin G1; AFG_2: aflatoxin G2; FB_1: fumonisin B_1; FB_2: fumonisin B_2; ZEN: zearalenone; DON: deoxynivalenol; NIV: nivalenol; OTA: ochratoxin A. HFB_1: Hydrolysed fumonisin B_1.

1.2.3 Characteristics and Toxic Effects of Main Mycotoxins

1.2.3.1 Aflatoxins

Since the discovery of AF in 1960 during the toxic outbreak in turkeys in England (LEESON; DIAZ; SUMMERS, 1995), more than 20 types of aflatoxin molecules have been identified, although the main types are B_1, B_2, G_1 and G_2 (HUSSEIN; BRASEL, 2001). Despite the structural similarities, the aflatoxins present different degrees of biological activity. AFB_1 is the most frequently compound found in cereals and presents the greatest toxigenic potential, followed by G_1, B_2 and G_2 (DIAZ, 2005). AFM_1 is a hydroxylated product found in milk as a result of biotransformation of AFB_1, also showing high toxicity similar to its parent compound (OLIVEIRA; ROSMANINHO; ROSIM, 2006). After oral ingestion, mycotoxins can be absorbed in different proportions, some of which are biotransformed before urinary or fecal excretion. The AFs are the most studied mycotoxins in relation to biotransformation standards and the biochemical basis of their toxic effects in animals and humans. The AFB_1 absorbed and its metabolites are excreted in urine and feces. AFB_1 is concentrated in the liver and, to a lesser extent, in the kidneys. Enzymes of the cytochrome family P450 (CYP), CYP1A2, CYP3A4 and CYP2A6, are the main responsible for the biotransformation of the absorbed AF (ESSIGMANN et al., 1982). These enzymes convert AFB_1 in its carcinogenic form, the AFB-8,9-epóxido, which is covalently bound to DNA and serum albumin, producing adducts of AFB_1-N^7-guanine and lysine, respectively (SABBIONI et al., 1987). In addition to being epoxidized, AFB_1 may also be bio transformed to generate several other derivatives. The main hydroxylated metabolites are AFM_1 and Q_1 (AFQ_1), whereas aflatoxin P_1 (AFP_1) and aflatoxicol (AFL) are demethylated and reduced metabolites, respectively.

Laboratory and production animals are very sensitive to the toxic effects of AF. In many species, males can be more susceptible than females, although, in general, youngsters can be considerably more sensitive than adults (OLIVEIRA et al., 2014). Acute toxic effects develop with fast deterioration of the animal's general state, loss of appetite, acute hepatitis, jaundice, hemorrhages and death (ABBAS, 2005). In addition, suppression of immunity by AFB_1 is observed in several animal species including turkeys, chicken, pigs, mice, guinea pigs and rabbits (OLIVEIRA et al., 2006).

In humans, the most important health effect of AF is hepatocellular carcinoma (HCC). This disease accounts for more than 80% of malignant primary tumors of the liver, being the 7th and 9th most common type of cancer worldwide affecting men and women, respectively. In Brazil, the incidence of HCC is 1: 100,000 inhabitants / year,

although this value is considered underestimated because of underreporting. The highest frequency occurs in the states of Amazonas, Bahia, Espírito Santo and Santa Catarina (PIMENTA; MASSABKI, 2010). In São Paulo, the incidence is slightly higher than the country average, affecting around 2: 100,000 inhabitants per year. Although a relatively rare tumor, HCCs are aggressive, and mortality rates reach significant levels, with around 312,000 deaths per year worldwide, and the maximum survival rates are 5% in 5 years. Liver carcinogenesis has been widely demonstrated - mainly in relation to AFB_1 - in many animal species, including fish, poultry, rodents, carnivores and primates (BUSBY; WOGAN, 1984). The association between AFB_1 and HCC is based on the toxin's ability to induce a specific mutation of the *p53* gene.

1.2.3.2 Fumonisins

FB toxins comprise the last group of mycotoxins discovered. Since their isolation in 1988, they have been associated with diseases in animals previously known as equine leukoencephalomalacia and pulmonary edema in swine (LEESON; DIAZ; SUMMERS, 1995). The main producer of FB is *F. verticillioides* (= *moniliforme*), although other *Fusarium* species are also known as FB producers, such as *F. proliferatum*, *F. nygamai*, *F. anthophilum*, *F. dlamini* and *F. napiforme* (SHEPHARD; THIEL; SYDENHAM, 1992). Currently, 28 molecular structures designated by the term fumonisin are known, but the toxin predominantly produced by strains of *F. verticillioides* is FB_1 (SEO; LEE, 1999). Besides FB_1, only FB_2 and FB_3 were detected when fumonisin production occurred under natural conditions (SHEPHARD; THIEL; SYDENHAM, 1992).

Studies with rodent hepatocytes have shown that FB block the formation of sphingolipids (RILLEY et al., 1996). This evidence supports the hypothesis that interruption of sphingolipid formation, resulting in accumulation of sphinganine and sphingosine, is the mechanism behind the acute toxicity and carcinogenicity effects of FB (NORRED; PLATTNER; CHAMBERLAIN, 1993). Sphingolipids are important for the maintenance of cell membrane integrity and regulation of cell surface receptors, ion pumps and other systems vital for cell function and survival. Immunosuppression is also seen as one of the major toxic effects of FB_1. In humans, dietary FB_1 has been associated as the causative agent of esophageal cancer, which is the 7th most important cause of cancer death among men in Brazil (IPCS, 2015). The incidence of this disease is three to four times higher in men than in women, and remarkable differences in incidence are found in small geographic areas and as a function of time. Previous studies have suggested that the consumption of contaminated corn products, especially polenta, is a

risk factor for esophageal cancer in rural areas in the State of Santa Catarina (IPCS, 2015). The Brazilian southern region, which comprises the states of Santa Catarina, Paraná and Rio Grande do Sul, has the highest incidence rate of esophageal cancer in the country, 11/100,000 inhabitants (INCA, 2015). Moreover, the southern and western regions of the state of Santa Catarina, where there is a large volume of corn production and high consumption of corn by-products (especially polenta in rural areas), also have the highest incidence of esophageal cancer.

1.2.3.3 Zearalenone

ZEN is an estrogenic substance derived from resorcilic acid, produced by species of *Fusarium*: *F. roseum* (*F. graminearum*), *F. culmorum*, *F. equisetum*, among others. Among the foods most susceptible to ZEN contamination, corn may be mentioned as it is an excellent substrate for fungal growth. However, other cereals can also be contaminated by the fungi and therefore present significant levels of ZEN, such as wheat, barley, oats, rye, sorghum and soybean (PITTET, 1998). After ingestion and subsequent absorption, ZEN can be reduced by the mammalian metabolism to its corresponding stereoisomeric hydroxy analogs, α-zearalenol and β-zearalenol (α- and β-ZEL), and also forms a conjugate of glucuronic acid, preferably, in the free phenolic 14-hydroxyl group (PFEIFFER et al., 2010). The most characteristic symptoms of ZEN poisoning are inflammation of the uterus, breast and vulva in female puberty, testicular atrophy and inflammation of the breasts in young males, and infertility in adult animals. Therefore, the effects of ZEN are predominantly estrogenic, mainly involving the urogenital system. Piglets are the most commonly affected animals, although dairy or meat cattle, laying hens, sheep and rodents also have different sensitivities to ZEN (HAGLER et al., 2001).

1.2.3.4. Trichothecenes

Trichothecenes are mycotoxins produced by different genera of fungi such as *Fusarium*, *Stachybotrys*, *Myrothecium*, *Trichotecium*, among others, since these fungi are also known as pathogens of grain-producing plants (MILLER, 1995). *Fusarium graminearum* and *F. culmorum* are the most important producer species (PITTET, 1998). Currently, almost 200 compounds called trichothecenes are known, but the most studied ones are DON (class B trichothecenes), T-2 and HT-2 toxins (class A trichotecenes). Studies have shown that the occurrence of DON is mainly observed in cereals, such as wheat, corn, barley and oats (ABBAS, 2005). Trichothecenes present in foods can be easily and quickly absorbed into the gastrointestinal tract of the exposed individual. In

the case of DON, animal studies have shown that the availability of 50-60% suggests efficient absorption. After ingestion, DON can be metabolized into de-epoxydioxynivalenol (DOM-1) by microorganisms in the intestinal flora of various animal species (LATTANZIO et al., 2011; MEKY et al., 2003; TURNER et al., 2008b). However, a recent study found that human fecal flora is also capable of metabolizing DON to DOM-1 (GRATZ; DUNCAN; RICHARDSON, 2013). The toxicity of trichothecenes in animals includes weight loss, food refusal, vomiting, diarrhea, bleeding, falling egg production, miscarriage, and death. DON is common in cereal grains in North America and Europe, where it has been associated with animal and human health problems. This toxin can be lethal if ingested in large quantities, and the intake of low to moderate levels may cause decreased performance and changes in immune function (CAST, 2003). T-2 and HT-2 cause alteration of multiple functions of the cell membrane, also inhibiting the synthesis of DNA and protein, leading to dematotoxic, immunotoxic and cytotoxic effects in several animal species (VAN DER FELS-KLERX; STRATAKOU, 2010).

1.2.3.5 Ochratoxin A

The ochratoxins were isolated from *Aspergillus ochraceus* (previously *A. alutaceus*), although they can also be produced by several species of *Aspergillus* and *Penicillium* (PITTET, 1998). The ochratoxin group consists of 7 components, but only OTA has been found as a natural contaminant in foodstuffs, especially in cereals. The target organ of the toxic action of OTA is the kidney, interfering in the macromolecule synthesis of renal parenchymal cells, including DNA, RNA and proteins. In addition, it affects the renal metabolism of carbohydrates, damaging the epithelium of the proximal renal tubules, which decreases the absorption of electrolytes and increases the excretion of water through osmotic diuresis (LEESON et al., 1995).

A summary of the toxicological characteristics of the main mycotoxins is presented in Table 3. In Brazil, there is little information on the occurrence of mycotoxin biomarkers in human urine samples, except for two studies in the state of São Paulo, one of them reported by Romero et al. (2010), who found 65% out of 65 urine samples from inhabitants of Piracicaba containing AFM_1 at concentrations ≥ 1.8 pg/mL (mean: 5.96 pg/mL). In the second study, Jager et al. (2014) observed AFM_1 in 39 urine samples (61%) of individuals from Pirassununga, at levels ranging from 0.19 to 12.7 pg/mg creatinine (mean: 1.2±2.0 pg/mg creatinine). However, there are no previous reports in Brazil on the evaluation of urinary biomarkers of human exposure to other important

mycotoxins such as OTA, ZEN and trichothecenes (DON, T-2 and HT-2), nor the evaluation of several mycotoxin biomarkers in the Brazilian population.

Table 3 - Mechanisms of action and toxic properties of some mycotoxins

Micotoxins	Primary events at the cellular level	Toxic properties	References
Aflatoxin B_1	Metabolic activation → AFB_1-8,9-epoxide → modification of the main cellular macromolecules	Hepatotoxic, immunosuppressive, carcinogenic (group 1, IARC), teratogenic, mutagenic	RILLEY; NORRED (1996), IARC (2002)
Aflatoxin M_1	Conjugation of epoxides → modification of cell macromolecules	Carcinogenic, mutagenic (group 2B, IARC)	(NEAL et al., 1998)
Fumonisin B_1	Inhibition of ceramide syntase → changes in the metabolism of sphingolipids → protein kinase activity, oxidative stress → oxidative damage of cell macromolecules	Equine leukoencephalomalacia, pulmonary edema in swine, hepatotoxic, nephrotoxic, immunosuppressive, carcinogenic (group 2B, IARC)	YAZAR; OMURTAG (2008), IARC (1993 RILLEY; NORRED (1996)
Deoxynivalenol	Inhibition of protein synthesis → disruption of cytokine regulation → cell death	Causes nausea, food refusal, vomiting, diarrhea, immunotoxic, IARC group 3	YAZAR; OMURTAG (2008), IARC (1993)
T-2 and HT-2	Alteration of multiple functions of the cell membrane → inhibits the synthesis of DNA and protein → apoptosis in different cell	Hematotoxic, immunotoxic, cytotoxic, dermal effects, IARC group 3	(VAN DER FELS-KLERX; STRATAKOU, 2010)
Zearalenone	It resembles 17 b-estradiol → binds to estrogen receptors on mammalian target cells	Disruption of hormonal control, IARC group 3	YAZAR; OMURTAG (2008), IARC (1993)
Ochratoxin A	Competition with phenylalanine and inhibition of Fe-dependent enzymes, inhibition of protein and DNA synthesis, mitochondrial transport system and transport of organic cations and anions, oxidative stress, DNA damage, affects glucose metabolism and Ca^{2+} homeostasis	Nephrotoxic, neurotoxic, hepatotoxic, affects blood coagulation, immunotoxic, carcinogenic (group 2B, IARC), teratogenic	IARC (1993)

IARC: International Agency for Research on Cancer.

Biomarkers have been evaluated for mycotoxins individually in human studies to define sensitivity, specificity, accuracy, and reliability parameters. Further validation in epidemiological studies evaluated intra and inter-individual variability, biomarker external dose relationship, and feasibility of use in broad population studies (GROOPMAN et al., 1992a, 1992b). Recently, liquid chromatography coupled to multi-

analyte mass spectrometry (LC-MS/MS) was successfully introduced into mycotoxin analyzes, including the evaluation of suitable biomarkers for human exposure assessment. The development of new analytical techniques has brought important contributions to the approach of multi-mycotoxin biomarkers, such as the measurement of a more realistic set of data on exposure, since it is expected that in real conditions a mixture of mycotoxins, and the potential application at risk of mycotoxins combined and their possible interaction effects. However, sample preparation remains a challenge for the development of multi-mycotoxin analysis methods because of their wide range of different chemical properties and their metabolites (OLIVEIRA, 2016).

Solfrizzo et al. (2004) analyzed FB_1, AFM_1, OTA, DON, DOM-1, and α- and β-zearalenol in a cleaning-based sample by multi-antibody immunoaffinity columns (IAC) and solid phase extraction columns (SPE). Subsequently, Warth et al. (2012) developed a multi-biomarker method based on a "dilution-and-shoot" approach for 15 mycotoxins, including FB_1, AFM_1, OTA, DON, DON-3-GlcA, DOM-1, and ZEN α- and β-zearalenol. Additional studies are still being conducted by some research groups in the world to develop multi-mycotoxin methods for biomarkers in urine, to provide an accurate estimate of the actual exposure to mycotoxins (OLIVEIRA, 2016).

1.2.4 Quality Assurance in Analytical Methods for Mycotoxins

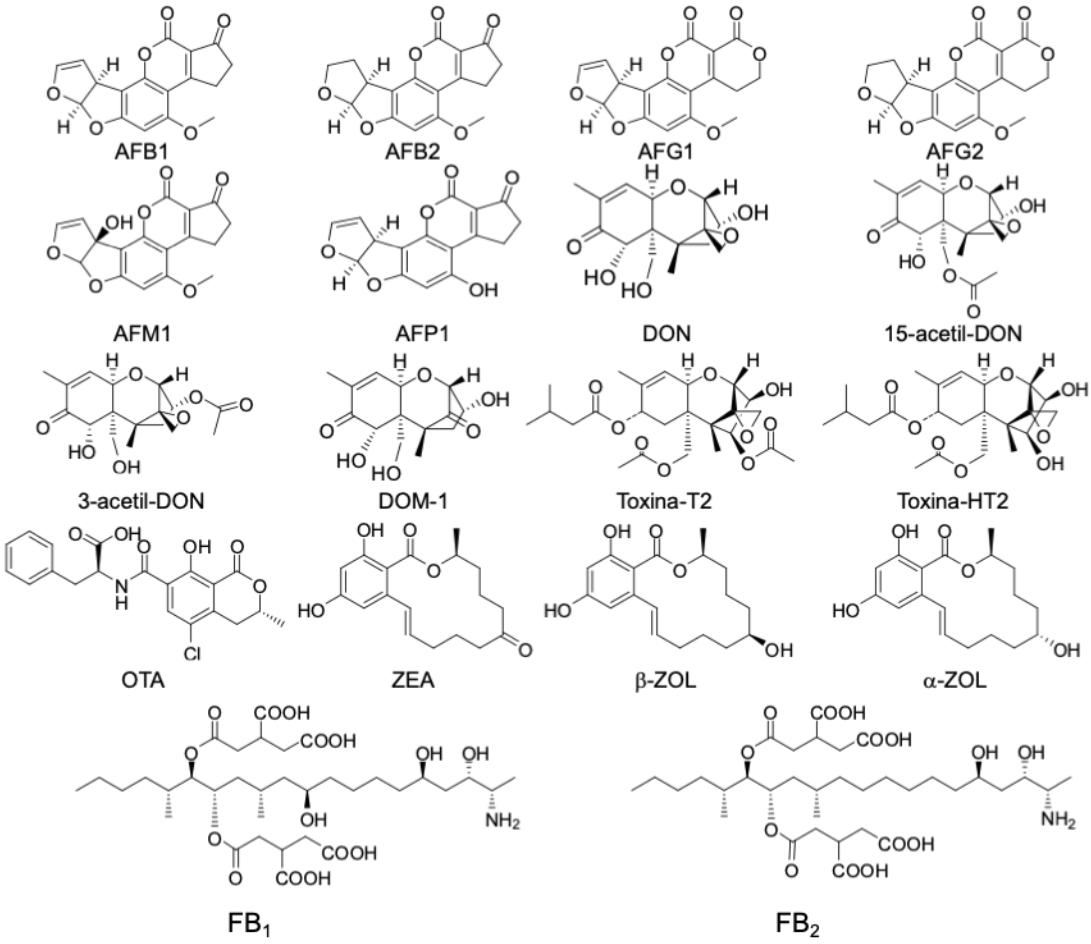
Different analytical methods are available for quantification of mycotoxins in food and biological samples, such as chromatographic methods (thin layer chromatography, TLC, high performance liquid chromatography (HPLC), immunological tests with specific antibodies ELISA, radioimmunoassay - RIA and immunohistochemical assays), as well as "hyphenated" techniques such as HPLC coupled to mass spectrometry (TURNER; SUBRAHMANYAM; PILETSKY, 2009). Each of these methods has advantages and disadvantages that affect the sensitivity and performance of the analyzes. Nevertheless, such analyses may present a great challenge, since mycotoxins may be present in very low concentrations in complex biological matrices, with a high diversity of biomolecules. Thus, these studies require the use of sensitive, selective, and robust analytical methods to provide accurate data on the composition of a given sample.

Currently, the LC-MS/MS technique operating with ionization source by electrospray (ESI) is undoubtedly the most successful analytical tool for the qualitative and qualitative determination of mycotoxins in natural samples (SONGSERMSAKUL; RAZZAZI-FAZELI, 2008; TURNER; SUBRAHMANYAM; PILETSKY, 2009). In particular, the use of the technique using sequential mass spectrometry (LC-MS / MS)

provides a pronounced gain in sensitivity and analytical selectivity, since MS / MS based methodologies use data regarding the relative molecular ion of a given analyte and its product ions thereby providing the maximum range of confidence in identifying a target analyte (HOFFMANN, 1996).

Modern mass spectrometers are increasingly versatile in the sense that it is possible to combine in a single instrument, different ionization sources and different analyzers. The great advantage is that the current equipment allows a more refined analytical development, thus allowing the analysis of a larger spectrum of molecules in a single instrument (WATSON; SPARKMAN, 2007). Figure 1 presents the chemical structure of mycotoxins studied in this Project.

Figure 1 - Chemical structure of mycotoxin analyzed in this study



Source: (Adapted from STEYN, 1995).

The analytical validation is fundamental to evaluate if an analytical method is adequate to the proposed objectives, to provide consistent results (BRITO et al., 2003). According to ANVISA Resolution RDC No. 27, dated May 17, 2012, the validation of

an analytical method should preferably be performed by chromatographic methods and should contain tests of precision, accuracy, calibration curve, matrix effect and selectivity.

Selectivity is the ability of the method to distinguish the substance in question from other components present in the sample. In the case of chromatographic methods, selectivity can be demonstrated by the absence of peaks at the same retention time of the analyte of interest in "blank" samples and the presence of peaks in fortified samples (LANÇAS, 2004). Thus, this parameter evaluates the degree of interference of other compounds of similar properties that may be present in the sample, ensuring that the peak response is exclusively from the compound of interest (ANVISA, 2012; RIBANI et al., 2004). Matrix Effect measurement is a study of selectivity that aims to investigate interferences caused by substances that make up the sample matrix generating, basically, the phenomena of decrease or amplification of the instrumental signal or instrumental response. The matrix effect study is necessary to use a calibration curve prepared in solvent, that is, with an unmatched calibration curve (BRAZIL, 2011b).

The linearity of a method is the ability to derive test results that are directly, or through a well-defined simple mathematical deduction, proportional to the concentration of analyte in the samples within a given range of application (RIBANI et al., 2004). The simplest way to observe linearity is by graphically plotting the response of a measure vs. the concentration or quantity of the known substance (calibration curve) defined by at least five points that do not include the zero point in the curve (INMETRO, 2003; RIBANI et al., 2004). The estimation of the coefficients of a curve from a set of experimental measurements can be performed using linear regression and calculating the correlation coefficient (R). A correlation coefficient greater than 0.999 is considered as evidence of an ideal fit of the data for the regression line (RIBANI et al., 2004). Anvisa (2003) recommends a minimum correlation coefficient equal to 0.99, although Inmetro (2003) indicate a value above 0.90. The linear range of the calibration curve should include the expected concentration range for the test sample (BRASIL, 2011b), meeting the requirements of precision and accuracy. Ribani et al. (2004) reported that the analytical curve points can be equally spaced over the concentration range of interest and that this range comprises 0-150% or 50-150% of the expected value depending on which of these options is most appropriate.

Accuracy indicates the proximity of independent assay results obtained by repeated multi-aliquot assays from a single matrix source under specified pre-set conditions, allowing the dispersion of results to be evaluated (ANVISA, 2012). The accuracy value

should be expressed in terms of imprecision and is usually calculated as a coefficient of variation (CV%). A lower accuracy is indicated by a high standard deviation (TURNER, 2009). Thus, values higher than 15% (fifteen percent), except for the LOQ, should not be admitted, for which values less than or equal to 20% (twenty percent) are allowed.

Accuracy relates to the agreement between the result of a test and a reference value. The most used processes to evaluate the accuracy are the use of reference materials, method comparison, recovery assays or standard addition (RIBANI et al., 2004). The recovery is defined as the percentage of the analyte of interest added that was recovered after the extraction process, with values outside the range of ± 15% (fifteen percent) of the nominal value, except for the LOQ, for which values outside the range of ± 20% (twenty percent) of the nominal value are allowed. Recovery should be evaluated at the expected concentration range for the compound of interest. This may be done by adding the substance in at least three different concentrations, for example, near the limit of quantification, close to the maximum concentration allowed by the test method and at a concentration close to the average range of the method use. It is also important to establish limits of detection (LOD) and quantification (LOQ). LOD is the lowest concentration of analyte present in a sample that the analytical procedure can reliably differentiate from background noise (ANVISA, 2003). The LOQ is usually calculated by comparing the measured signals of the sample with low known concentrations of the analyte with the blank, establishing the minimum concentration at which the analyte can be quantified (BRITO et al., 2003).

1.2.5 Regulations for mycotoxins

Brazilian regulations for mycotoxins in foods were revised in 2011, to include maximum permitted levels (MPL) for OTA, FB, ZEN and DON in several food products along with already established MPL for AF. For corn products, MPL established for total AF ($B_1 + B_2 + G_1 + G_2$), OTA, FB ($B_1 + B_2$), ZEN and DON were 20, 10, 1500, 150 and 750 μg/kg, respectively (ANVISA, 2011a). However, the MPL for total AF is 5 μg/kg for rice, beans and wheat flour. The MPL for DON and OTA in rice, beans, wheat flour, corn flour and corn meal are 750 μg/kg and 10 μg/kg, respectively. For ZEN, the MPL is 100 μg/kg for rice and wheat flour.

Compared with the MPL adopted in Brazil, lower mycotoxin levels were established in European countries for the total AF (2 μg/kg) in all products derived from cereals including processed cereal products. However, similar MPL values are in force in Europe for DON (750 μg/kg) in cereals, cereal flour, bran and germ as end products sold for

direct human consumption. European regulations also adopted MPL of 5 μg/kg of OTA in rice and beans, and 3 μg/kg for wheat flour and corn products. ZEN has a MPL of 75 μg/kg for all types foods, and total FB of 1,000 μg/kg as MPL for corn meal and corn flour (VERSTRAETE, 2008).

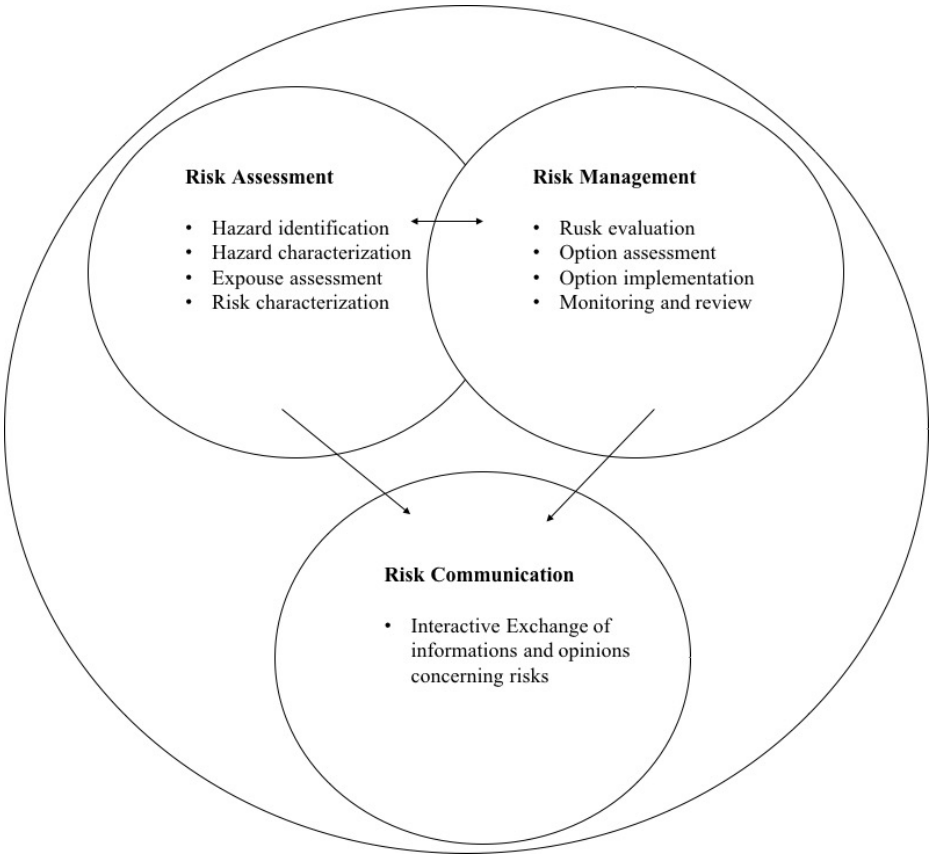
European Union regulations also established MPL for mycotoxins in feed, such as AFB_1 in animal feed for poultry (10 μg/kg) and for dairy cattle (5 μg/kg), DON in feed ingredients (8,000 μg/kg), ZEN in animal feed for dairy cattle (500 μg/kg), OTA in grain and coproducts for feed ingredients (250 μg/kg), and total FB for poultry feed (20,000 μg/kg) and feed for dairy cattle (50,000 μg/kg). In Brazil, the only regulation available refers to a maximum recommended limit of 50 μg/kg for total AF in feed ingredients (OLIVEIRA, 2014).

1.2.6 Risk analysis for mycotoxins

The *Codex Alimentarius* Commission (CAC) has defined Risk Analysis by a process consisting of three components: risk assessment, risk management and risk communication (FAO/WHO, 2009). The Figure 2 shows of the risk analysis paradigm. The separation between risk assessors and risk managers is critical to ensuring the scientific objectivity of the risk assessment process, which constitutes the basis for establishing science-based standards such as maximum limits for contaminants in foodstuffs (FAO/WHO, 2009).

A risk analysis allows authorities to identify the control points along the food chain, studying measures to be applied, weighing costs and increasing benefits. Risk analysis is generally used to obtain information about the level of risk of a particular contaminant in food by helping government agencies decide what actions to take, such as the maximum allowable amount of a contaminant in the food. Therefore, the comparison with the allowable limit of a certain contaminant is the preferred way of assessing possible links between food chain hazards and actual risks to human health, taking into account a wide range of inputs for decision-making on appropriate control measures (FAO/WHO, 2006).

Figure 2 - Risk analysis



Source: (Adapted from FAO/WHO, 2009).

1.2.6.1 Risk Assessment

Risk assessment is the main task of Risk Analysis, with the main function being to make decisions to protect health in view of scientific uncertainty. The risk assessment of food chemicals is generally described by characterizing potential hazards and the associated risks to human health resulting from exposure to these products present in food over a given period of time (ASSUNÇÃO et al., 2015; CANO-SANCHO et al., 2013; DENG et al., 2018; FAO/WHO, 2009; SERRANO et al., 2012). Risk assessment is divided into four stages: hazard identification, hazard characterization, exposure assessment, and risk characterization.

1.2.6.1.1 Hazard identification

Hazard identification starts with the identification of the type and nature of adverse effects that an agent can cause in an organism, system, or population (IPCS, 2004). The

Hazard identification is the first stage of four steps in risk assessment, with the purpose of evaluating the weight of evidence for adverse health effects, based on assessment of all available data on toxicity and mode of action (DE BOEVRE et al., 2013; DENG et al., 2018; HEYNDRICKX et al., 2015). It is designed to primarily address the nature of any health hazard to humans that an agent may pose and the circumstances under which an identified hazard may be expressed. Hazard identification is based on analyses of a variety of data, ranging from observations in humans or domestic animals and studies in laboratory animals and *in vitro* laboratory studies through to analysis of structure–activity relationships (FAO/WHO, 2009).

1.2.6.1.2 Hazard characterization

Hazard characterization is the qualitative and, wherever possible, quantitative description of the inherent properties of an agent or situation having the potential to cause adverse effects, and for this, should include a dose–response assessment and its attendant uncertainties (IPCS, 2004). Hazard characterization is the second stage of steps in risk assessment, describing the relationship between the administered dose of, or exposure to, a chemical and the incidence of an adverse health effect (FAO/WHO, 2009).

The critical effect is the first adverse effect observed as the dose or exposure is increased. The characterization of the hazard begins when the critical effect is observed, thus considering the beginning of the toxic effect. With this dose-response observation, a health-based guideline value is established, being an acceptable daily intake (ADI) for additives or wastes, and a tolerable intake (TI) for contaminants. When a substance is assessed to be of very low toxicity, based on the biological and toxicological data, and the total dietary intake of the substance does not represent a hazard, the ADI do not need to be specified (FAO/WHO, 2009).

1.2.6.1.3 Exposure assessment

Exposure assessment is defined by CAC as "The qualitative and/or quantitative evaluation of the likely intake of chemical agents via food as well as exposure from other sources if relevant" (FAO/WHO, 2008). Chemicals present in food have an assessment of food exposure considering the occurrence and concentrations present in the diet through food consumption and the probability of consumers ingesting large quantities of the food and the chemical in question (DE BOEVRE et al., 2013; DENG et al., 2018; GILBERT et al., 2001; GONG et al., 2008; TURNER et al., 2009). A range of intake or exposure estimate can be provided considering different types of consumers, such as

average or high consumers for a particular type of food, Additionally, exposure estimates may be categorized in subgroups of the population, such as children and adults (FAO/WHO, 2009).

According Kuiper-Goodman (1995), mycotoxins can be considered as natural contaminants of foodstuffs. In this case, food ingestion is the considered main route of exposure to mycotoxins and, consequently, a deep knowledge within this area is fundamental for an accurate risk assessment (ASSUNÇÃO et al., 2015). Mycotoxin exposure assessment studies cover a set of qualification and quantification data, requiring representative sampling, for example selecting the most susceptible foods and a precise analytical method, with low detection limits, in order to obtain exposure estimates with a low level of uncertainty (ASSUNÇÃO et al., 2015; CANO-SANCHO et al., 2013). Several studies have been performed to evaluate the exposure assessment to mycotoxins for humans, as indicated in Table 4. However, there are few published works on the evaluation of health risk using approaches that consider the simultaneous exposure to different mycotoxins.

Table 4 - Studies on the human exposure assessments to dietary mycotoxins

continue

Local	Mycotoxins	Data source	Reference
Brazil	Food: AFB_1, AFM_1, and AFB_1-lysine Urine: AFM_1 and AFB_1-N^7-guanine	Food samples and urinary biomarkers	(JAGER et al., 2016)
South Africa	Food: AFs, FB1, FB2, DON, ZEN and OTA Urine: FB1, FB2, AFM1, OTA, DON, DON-3-GlcA, DON-15-GlcA, DOM-1, NIV, T-2 toxin, HT-2 toxin, ZEN, ZEN-14-GlcA, α-ZEL and β-ZEL	Food samples and urinary biomarkers	(SHEPHARD et al., 2013)
United Kingdom	DON	Food samples and urinary biomarkers	(TURNER et al., 2010b)
United Kingdom	DON	Food samples and urinary biomarkers	(TURNER et al., 2008a)
Bangladesh, Germany, and Haiti	DON, DON-GlcA, FB_1, AFM_1 and α-ZEL	Urinary biomarkers	(GERDING et al., 2015)
Italy	DON, AFM1, FB1, α-ZEL, β-ZEL, ZEN and OTA	Urinary biomarkers	(SOLFRIZZO; GAMBACORTA; VISCONTI, 2014)
Germany	ZAN, ZEN, β-ZEL, α-ZEL, AFM_1, AFB_1, AFB_2, AFG_1, AFG_2, DON, FB_1, FB_2, OTA, OTα, T-2, HT-2	Urinary biomarkers	(GERDING; CRAMER; HUMPF, 2014)

Table 4 - Studies on the human exposure assessments to dietary mycotoxins

conclusion

Local	Mycotoxins	Data source	Reference
Thailand	AFM_1, FB_1, FB_2, OTA, DON, DON-3-GlcA, DOM-1, NIV, T-2 toxin, HT-2 toxin, ZEN, ZEN-14-GlcA, α-ZEL and β-ZEL	Urinary biomarkers	(WARTH et al., 2014)
France	DON, DOM-1	Urinary biomarkers	(TURNER et al., 2010a)
Brazil	FB_1	Food samples	(BORDIN et al., 2015)
Brazil	AFB_1, AFB_2, AFG_1, AFG_2 and AFM_1	Food samples	(JAGER et al., 2013)
Brazil	DON	Food samples	(SANTOS et al., 2013)
Brazil	FB_1, FB_2	Food samples	(MARTINS et al., 2012a)

AFB_1: aflatoxin B1; AFB_2: aflatoxin B2; AFG_1: aflatoxin G1; AFG_2: aflatoxin G2; AFM_1: aflatoxin M1; FB_1: fumonisin B1; FB_2: fumonisin B2; FB_3: fumonisin B3; ZEN: zearalenone; DON: deoxynivalenol; NIV: nivalenol; OTA: ochratoxin A; DOM-1: deepoxy-deoxynivalenol; DON-GlcA: deoxynivalenol glucuronide; ZEN-14-GlcA: zearalenone-14-O-glucuronide; α-ZEL: α-zearalenol; β-ZEL: β-zearalenol.

The intake levels determined in exposure assessments can be compared with reference values for mycotoxins, or maximum tolerable daily intake (TDI). FAO/WHO (2011a) established a TDI for total FB (FB_1, B_2 and B_3) of 2 μg/kg body weight (b.w.) per day. For OTA, the TDI is 0.016 μg/kg b.w./day (WHO/FAO, 2007), while TDI values for DON and ZEN are 1.0 μg/kg b.w./day (WHO/FAO, 2011b) and 0.25 μg/kg b.w. /day (EFSA, 2016).

The Hazard Quotient (HQ) is obtained by comparing their respective reference dose (TDI) with the estimated exposure, evaluating whether the exposure level is tolerable or not. In this context, HQ value < 1 indicates a tolerable exposure level, while HQ > 1 indicates a non-tolerable exposure level (EFSA, 2013). For substances that are genotoxic and carcinogenic, such as aflatoxins, the Margin of Exposure (MOE) is used instead of HQ, which is the ratio between the amount of the substance that produces a measurable effect in laboratory or human animals and the estimated human exposure (FAO/WHO, 2009; EFSA, 2013) The Scientific Committee of EFSA considers that MoE values of 10,000 or more, when based on a benchmark dose lower confidence limit 10 (BMDL10), and taking into account overall uncertainties in the interpretation, are considered of low concern from a public health perspective. EFSA's Scientific Committee also notes that the magnitude of the MOE only indicates a level of concern and does not quantify risk (EFSA, 2012, 2013).

1.2.6.1.4 Risk characterization

Risk characterization is the qualitative and, wherever possible, quantitative

determination, including attendant uncertainties, of the probability of occurrence of known and potential adverse effects of an agent in a given organism, system, or subpopulation, under defined exposure conditions (IPCS, 2004). Risk characterization is the final step of risk assessment is, if restricted to the population of consumers only (FAO/WHO, 2008). The appropriate recommendation should be made for risk management decision-making through the information obtained from consumption and exposure, obtaining estimates of the potential risk to human health in different exposure scenarios including all key health risk scenarios human (FAO/WHO, 2009).

The risk characterization statement should be a clear explanation of any uncertainties in the risk assessment resulting from gaps in the science base, also it should include, where relevant, information on susceptible subpopulations, including those with greater potential exposure or specific predisposing physiological conditions or genetic factors. The advice to risk managers can be qualitative or quantitative. Qualitative information include evidence that the chemical is of no toxicological concern; evidence that the chemical is safe for specified uses; and recommendations to avoid, minimize or reduce exposure. Thus, the quantitative information may include a comparison of dietary exposures with health-based values; estimates of risks at different levels of dietary exposure; risks at minimum and maximum dietary intakes; and margins of exposure (FAO/WHO, 2009).

The risk characterization of toxic compounds has been approached in different ways depending on its genotoxic and carcinogenic potential. In the case of mycotoxins, the risk characterization has been done through a health-oriented guideline value (TDI), compared with estimates of dietary exposure (Probable Daily Intake) (EFSA, 2013).

1.2.6.2 Risk management

Risk managers are responsible for deciding whether a risk assessment is necessary and evaluating other factors relevant to protecting the health of consumers with appropriate prevention and control options. If necessary, they organize risk assessment by performing tasks such as describing the purpose of risk assessment and food safety issues to be addressed, establishing a policy and providing the resources needed to carry out the work (FAO/WHO, 2009).

1.2.6.3 Risk communication

The risk communication is an interactive exchange of information and opinions on the process of risk analysis of management options and actions among risk managers,

consumers and other stakeholders, including explanation of the results of the risk assessment and the basis of risk management decisions (FAO/WHO, 2009).

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2 Chapter I: **Biomonitoring of mycotoxin exposure using urinary biomarker approaches: a review**

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Abstract

Mycotoxin contamination in food products is a serious human health concern. Estimation of human exposure to dietary mycotoxins is important for the risk assessment of these food contaminants. Available data on toxicokinetics of several mycotoxins in animal models and some human studies indicate that exposure to mycotoxins can be measured by biomarkers in several bio-specimens, especially in urine. This paper reviews the toxicokinetic basis of mycotoxin biomarkers in urine and recent data on human biomonitoring of aflatoxins, ochratoxin A, fumonisin, zearalenone, and deoxynivalenol using urinary biomarkers. Multi-mycotoxin biomarker approaches for assessment of combined exposure to multiple mycotoxins are also discussed.

Keywords: Exposure assessment. Mycotoxins. Human biomonitoring. Urine. Biomarkers.

2.1 Introduction

Mycotoxins are defined as secondary metabolites released by mycotoxigenic fungi that are able to grow in foods throughout the production chain. Mycotoxins can cause adverse effects on several animal species, including humans (ZAIN, 2011). The main toxigenic fungi are found in the genera *Aspergillus*, *Penicillium*, and *Fusarium*, and the primary classes of mycotoxin produced by these genera are aflatoxins (*Aspergillus*), ochratoxin A (*Aspergillus* and *Penicillium*), fumonisins, zearalenone and trichothecenes including deoxynivalenol (*Fusarium*) (BRYDEN, 2007). Co-prevalence of mycotoxin mixtures in food products is also possible, which increases public health risks due to the possible synergistic interactions causing multiple toxic effects (ALASSANE-KPEMBI et al., 2017; ASSUNÇÃO; SILVA; ALVITO, 2016).

Diseases caused by mycotoxins in humans and animals are called mycotoxicoses and are diffuse syndromes that cause lesions in organs such as liver, kidneys, epithelial tissue (skin and mucous membranes), and central nervous system, depending on the type of toxin (DIAZ, 2005). Additionally, some mycotoxins like aflatoxins (AF) are categorized as Group 1, human carcinogen, by the International Agency for Research on Cancer (2002). Others, such as ochratoxin A (OTA) and fumonisins (FB), are classified in Group 2B, probable human carcinogens (OSTRY, et al. 2017). Zearalenone (ZEN) binds competitively to estrogen receptors, leading to estrogenic abnormalities in animals and generative syndromes, especially in pigs and humans (BORUTOVA et al., 2012; SCHOEVERS et al., 2012). Deoxynivalenol (DON, also named vomitoxin) can cause

reactions such as nausea, diarrhea, reduced nutritional efficiency, gastrointestinal tract injuries, weight loss, and immune system regulation problems (MEKY et al., 2001; WU et al., 2015).

One of the most important aspects in risk analysis of chemical substances is to determine the degree of human exposure, which is particularly challenging for contaminants found in foodstuffs, such as mycotoxins. Exposure estimates can be based on data on the consumption of contaminated foodstuffs and on the average occurrence of the toxin (ASSUNÇÃO et al., 2015). These classical approaches provide useful data, although they are not always ideal. Exposure biomarkers have been proposed to improve direct exposure assessment to dietary mycotoxins. Biomarkers of exposure indicate the presence and magnitude of exposure to the agent by means of the quantification of the specific compound, its metabolite(s) or interaction products in a body compartment or fluid (GROOPMAN; KENSLER, 1999).

Available data on toxicokinetics of several mycotoxins in animal models and some human studies indicate that exposure to mycotoxins can be measured by biomarkers in bio-specimens, especially in urine (HEYNDRICKX et al., 2015). In recent years, liquid chromatography tandem mass spectrometry (LC-MS/MS) based on multi-analyte methods have been successfully introduced in mycotoxin analysis, including in the evaluation of suitable biomarkers for the assessment of human exposure to mycotoxins (HEYNDRICKX et al., 2015; SOLFRIZZO et al., 2011; WARTH et al., 2014). Thus, the objective of this paper is to review the toxicokinetic basis for mycotoxin biomarkers in urine and the available data published in the past 10 years (2009 to date) on human biomonitoring of the most relevant mycotoxins for human health using single and multi-mycotoxin urinary biomarker approaches.

2.2 Search strategy

Relevant studies published from 2009 to November 2018 were selected after a systematic literature search in PubMed, Science Direct, and Google Scholar databases, using the following key terms: “Aflatoxin” OR “Ochratoxin A” OR “Fumonisin” OR “Zearalenone” OR “Deoxynivalenol” OR “Mycotoxins” AND “Urine” OR “Human urine” OR Urinary excretion” OR “Biomarker” OR “Biomonitoring” OR "Exposure". First the full texts of potentially eligible articles were downloaded and examined twice based on criteria for inclusion and final eligibility. Inclusion criteria were: (1) article published in English; (2) full-text article available; (3) descriptive cross-sectional data; (4) original research surveys conducted with human urine; (5) number of samples

analyzed > 30; (6) expression of exact number of positive samples; (7) description of accurate analytical methods. In total, 57 articles fulfilled the inclusion criteria and were analyzed in this review.

2.3 Toxicokinectic basis for urinary biomarkers of main mycotoxins

2.3.1 Aflatoxins

Twenty different types of AF have been identified so far, although only AFB_1, AFB_2, AFG_1 and AFG_2 have frequently been found as natural contaminants of food products (HUSSEIN; BRASEL, 2001). AFB_1 is the most toxic compound. After being ingested it is primarily biotransformed in the liver by the cytochrome P450 system, generating hydroxylated metabolites, such as aflatoxins M_1 (AFM_1) Q_1, P_1, and B_{2a} (SUN et al., 2016). During biotransformation processes, AFB_1-N^7-guanine adduct (JAGER et al., 2016) is also formed by the binding of AFB-8,9-epoxide to DNA. Importantly, AFM_1 is the main hydroxylated compound originated from ingested AFB_1 and is excreted in urine and milk of women and lactating animals exposed to aflatoxins in contaminated food (CAMPAGNOLLO et al., 2016).

Although all AF metabolites can be excreted in urine, animal studies clearly indicate a dose-response relationship between AFM_1 or AFB_1-N^7-guanine levels in urine and incidence of liver tumors (HSIEH; ATKINSON, 1991; QIAN et al., 1994). In human populations, several studies reported the incidence of AF metabolites in urine, especially in areas with high AF contamination of foods, such Gambia (GROOPMAN et al., 1992a), Egypt, and Guinea (POLYCHRONAKI et al., 2008). Percentage of AF metabolites excreted in urine were 4.4% and 7.6% of AFB_1 ingested by women and men, respectively (GROOPMAN et al., 1992a). Zhu et al. (1987) concluded that AFM_1 excreted in urine ranged from 1.23 to 2.18% of total AFB_1 consumed for men, and from 1.30 to 1.78% for women. In piglets, about 2.5% of AFM_1 was excreted as urinary biomarker during 24 hours (GAMBACORTA et al., 2013), which is similar to humans percentages as described by Zhu et al. (1987).

2.3.2 Ochratoxin A

The most important dietary sources of OTA exposure are cereals used as human food and some beverages, including wine, fruit juices, coffee, and beer (GONÇALVES et al., 2018). It is estimated that cereals and beverages contribute with 50-70% and 7-15% of total OTA dietary intake, respectively (EUROPEAN COMMISSION, 2006). OTA is also found in food products of animal origin, such as poultry and pork, as a result of

animal intake of contaminated feed (JØRGENSEN, 1998). The kidneys are the main organs affected by OTA due to its nephrotoxic effects; it also causes liver damage in monogastric animals. Its immunosuppressive properties lead to higher susceptibility to infections, and it has teratogenic and carcinogenic properties. Protein synthesis may be inhibited by OTA competition with phenylalanine in the reaction catalyzed by phenylalanyl-tRNA synthase (RIZZO; ESKOLA; ATROSHI, 2002).

Around 40-66% OTA is absorbed in the upper gastrointestinal tract of several animal species. In the blood, OTA can remain linked to proteins and reach the kidneys, muscles, and liver, with estimated half-life of 35 days (BORCHERS et al., 2010). OTA demonstrates a plasma-protein binding potential of up to 99% (HAGELBERG; HULT; FUCHS, 1989). The long half-life of albumin is the major reason for the presence of OTA in human serum (PETZINGER; ZIEGLER, 2000). The toxin is eliminated by hepatic first passage, and it is removed in the bile before it enters the circulation. Therefore, it is not removed by glomerular filtration, but rather eliminated directly into the urine (GROVES; MORALES; WRIGHT, 1998). Percentages of OTA excreted in urine are nearly 50% of the OTA ingested by humans (SCHLATTER; STUDERROHR; RASONYI, 1996). Part of the OTA toxin is subjected to a hepatic first pass ilimination and is removed by the bile before it can enter blood circulation, therefore is not removed by glomerular filtration but is elimination to the urine. The principal organ afected for OTA are the Kidneys, where it causes nephrotoxic effects, and liver damage in monogastric animals, with immunosuppressive properties resulting in higher susceptibility to infections, and it is teratogenic and carcinogenic. Furthermore, the protein synthesis may be a inhibit by competing with phenylalanine in the phenylalanyl- tRNA synthase-catalysed reaction. Ochratoxin A demonstrates a plasma-protein binding potential of up to 99% (HAGELBERG; HULT; FUCHS, 1989). The long half-life of albumin is the major reason for the presence of OTA in human serum (PETZINGER; ZIEGLER, 2000). The toxin is eliminated by hepatic first passage, and it is removed in the bile before it enters the circulation. Therefore, it is not removed by glomerular filtration, but rather eliminated directly into the urine (GROVES; MORALES; WRIGHT, 1998). Percentages of OTA excreted in urine are nearly 50% of the OTA ingested by humans (SCHLATTER; STUDERROHR; RASONYI, 1996).

2.3.3 Fumonisins

Twenty-eight structurally related FB have been isolated and identified, although FB_1 is the most predominant and most toxic fumonisin form produced by fungi

(MAGAN; OLSEN, 2006). FB_1 causes leukoencephalomalacia in horses (MARASAS et al., 1988) and pulmonary edema in swine (HASCHEK et al., 1992). It also exhibits nephrotoxic, hepatotoxic, and hepatocarcinogenic effects in rats (WAN NORHASIMA et al., 2009), and is associated with esophageal cancer and neural tube defects in humans (MARASAS, 2001; MISSMER et al., 2006). Epidemiological studies conducted in several countries revealed that the incidence of human esophageal cancer correlates with the occurrence of FB in food products, which was also associated with a low socioeconomic status and less varied diets based on corn or wheat (WORLD HEALTH ORGANIZATION, 2000).

Delongchamp and Young (2001) extrapolated to humans the toxicokinetically determined half-life after FB_1 administration in animal models, and estimated that the half-life in a 70-kg person is 128 min. Tested oral bioavailability of FB_1 in several animal species is lower than 6% (MARTINEZ-LARRANAGA, 1999), with less than 2% recovered in urine (SHEPHARD; VAN DER WESTHUIZEN; SEWRAM, 2007). Gambacorta et al. (2013) studied urinary biomarkers in piglets and the percentage of FB_1 excreted in urine was 2.6%. However, FB_1 excreted in human urine was 0.5% of the total mycotoxin ingested (RILEY et al., 2012).

2.3.4 Zearalenone

After oral intake of contaminated foods, ZEN is rapidly absorbed and then metabolized in the intestines or liver. There, the toxin undergoes hydroxylation with production of several metabolites that are usually conjugated with glucuronic acid, thus facilitating their excretion in urine (BORCHERS et al., 2010). ZEN is primarily degraded into α-zearalenol (α-ZEL) and β-zearalenol (β-ZEL), which undergo additional reduction into α-zearalanol (α-ZAL) and β-zearalanol (β-ZAL). The α-ZAL is metabolized into its isomer β-ZAL and, to a lesser extent, into zearalanone (ZAN). These compounds are, then, partially conjugated with sulfonic or glucuronic acid and excreted in the urine (BERNHOFT et al., 2001; KUIPER-GOODMAN et al., 1987; ZINEDINE et al., 2007). In humans, urinary excretion of ZEN is approximately 10% of the administered dose, with ZEN glucuronides and α-ZEL as the main metabolites, along with minor amounts of β -ZEL (FLECK et al., 2016). Urinary biomarkers in piglets show excretion 36.8% of dietary ZEN in 24 hours (GAMBACORTA et al., 2013). Half-life is approximately 3 days after ZEN administration (UENO et al., 1977; OLSEN et al., 1985; BERNHOFT et al., 2001).

A study proved that ZEN forms complex with human serum albumin, and

suggested that this complex was a stable and possibly biologically relevant interaction (POÓR et al., 2017). Videmann et al. (2008) suggested that the conversion of ZEA into α-ZEL may be a bioactivation reaction, while the formation of β-ZEL would be a detoxification reaction. The authors observed that intestinal Caco-2 cells produced predominantly α-ZEL and that was quantitatively the main metabolite at the basolateral side of the cells. In this case, human susceptibility to the undesirable effects of ZEA is increased by its intestinal metabolism.

2.3.5 Deoxynivalenol

Animal studies have shown that the bioavailability of DON is 50-60%, indicating efficient absorption (GOYARTS; DÄNICKE, 2006; MEKY et al., 2003; VIDAL et al., 2018). DON is quickly absorbed in the upper gastrointestinal tract and is biotransformed by different intestinal microorganisms in various animal species. Animal intestines show a wide number of DON-degrading bacteria that anaerobically transform it into de-epoxy-deoxynivalenol (DOM-1) (GRENIER; APPLEGATE, 2013; TURNER et al., 2008), although a recent study found that human fecal microbiota is able to metabolize DON into DOM-1 (GRATZ; DUNCAN; RICHARDSON, 2013). DON may be acetylated by the loss of side groups C4, C15 or C8, resulting in 15-acetyl-deoxynivalenol (15-Ac-DON) and 3-acetyl-deoxynivalenol (3-Ac-DON), which are less toxic compared with the parent compound (MAUL et al., 2015). Ruminants show increased tolerance to DON due to higher intra-ruminal microbial detoxification of DON into de-epoxy-deoxynivalenol (DOM-1) (WU et al., 2010).

DON is mainly eliminated by conjugation with glucuronic acid followed by urinary and fecal excretion (TURNER et al., 2008; WARTH et al., 2013; WU et al., 2010). Major metabolites generated with this conjugation are DON 15-glucuronide (DON 15-GlcA) and DON 3-glucuronide (DON 3-GlcA), besides glucuronidation products and isomeric DON such as DON 8-glucuronide (DON 8-GlcA), iso-DON 3-glucuronide (iso-DON 3-GlCA), and iso-DON 8-glucuronide (iso-DON 8-GlCA) (PESTKA et al., 2017). Generally, DON half-life is reasonably short, and the majority of DON and DON metabolites are excreted within 24h (PESTKA; SMOLINSKI 2005; VIDAL et al., 2018). The estimated average transfer of DON to urine is 50% (SHEPHARD et al., 2013) and 72.3% (TURNER et al., 2010a). However, a recent study showed that the total non-metabolized DON plus its metabolites (DON 15-GlcA and DON 3-GlcA) recovered in urine of men and women after 24 hours were 44.6 ± 13.9% and 72.7 ± 13.5% of the ingested mycotoxin, respectively (VIDAL et al., 2018).

2.4 Worldwide biomonitoring of mycotoxins in human urine

In human studies, urinary biomarkers have been proposed for individual mycotoxins to define sensitivity, specificity, accuracy, and reliability parameters (AKDEMIR et al., 2010). Therefore, a successful analytical method for determination of residual mycotoxins in urine samples should be robust and sensitive, and all techniques should be reproducible to a high level, with relevant and easy-to-analyze results (TURNER; SUBRAHMANY; PILETSKY, 2009). For some mycotoxins, such as AF and FB, further validation has been achieved in animal models and in epidemiological studies evaluating intra and inter-individual variability, biomarker external dose relationship, and feasibility of use in broad population studies (GROOPMAN et al., 1992b, 1992c).

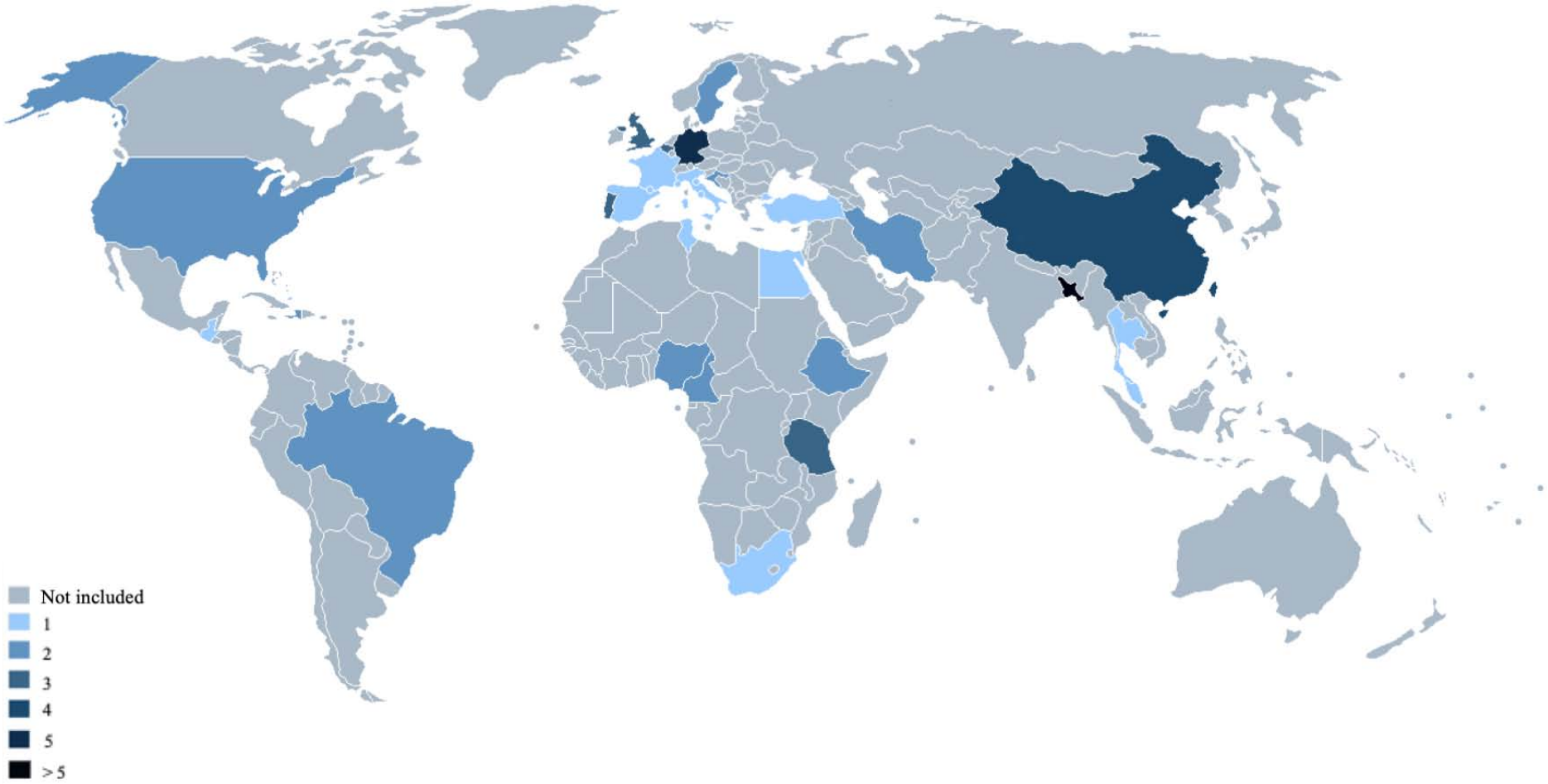
The development of LC-MS/MS analytical techniques using a multi-mycotoxin biomarker approach brought important contributions for exposure assessment, such as the measurement of more realistic data (since a mixture of mycotoxins is expected to occur under field conditions), and the potential application of multi-mycotoxin data in risk assessment of combined mycotoxins and their possible interaction effects. One of the first studies describing a multi-mycotoxin approach in urine was conducted by Solfrizzo et al. (2011). The authors analyzed FB_1, AFM_1, OTA, DON, DOM-1, and α- and β-ZEL based on sample clean-up by multi-antibody immunoaffinity columns (IAC) and solid phase extraction (SPE) columns. Subsequently, Warth et al. (2012) developed a multi-biomarker method based on a ‘dilute-and-shoot’ approach (no sample preparation other than centrifugation and dilution) for 15 mycotoxins, including FB_1, AFM_1, OTA, DON, DON-3-GlcA, DOM-1, ZEA, and α- and β-ZEL. Latter studies proposed analytical methods para other mycotoxins that are not officially regulated in food products, such as Enniatins (EN), Beauvericin (BEA), Nivalenol (NIV), Citrinin (CIT), Alternariol (AOH), Fusarenon X (FUS-X), Diacetoxyscirpenol (DAS), Neosolaniol (NEO), and Sterigmatocystin (STER) (HENDRICKX et al., 2015; HUYBRECHTS et al., 2015; SARKANJ et al., 2018).

Figure 3 presents an overview of the studies conducted worldwide in the past 10 years (2009 to date) regarding human exposure to mycotoxins using single or multi-analyte urinary biomarker approaches. Bangladesh presented the highest number of urinary biomarker studies in the last years, with seven reports. Germany was the second country with more urinary biomarker data, with five reports, followed by China, with four studies. Europe is the continent with the greatest number of countries (N = 23) that provided data on mycotoxin biomarkers in human urine. Asian and African countries

presented 16 and 7 studies, respectively. America had the lowest number of countries (N = 4) studying urinary biomarkers in humans, with two reports from Brazil, two from the United States, two from Haiti, and one from Guatemala.

Table 5 presents data on mycotoxin biomarkers in human urine from studies conducted in African, American. Asian and European countries in the past 10 years. In African countries, several mycotoxins were frequently reported in high concentration in the urine of individual in Cameroon (ABIA et al., 2013; EDIAGE et al., 2013), South Africa (SHEPHARD et al., 2013), and Nigeria (EZEKIEL et al., 2014; SARKANJ et al., 2018). FB_1 was analyzed in eight studies, with percentages of positives samples varying from 3% to 100%. Ediage et al. (2013) reported levels of FB_1 up to 48,000 ng/L in Cameroon. Ezekiel et al. (2014) reported maximum levels of FB_1 at 12,800 ng/L in Nigeria and Sarkanj et al. (2018) confirmed the presence of FB_1 in Nigerian urine with levels up to 14,880 ng/L. These studies revealed that FB_1 exposure could constitute a probable health hazard in Africa, especially if it is taken into account that only 0.5% of the total ingested of FB_1 is excreted in urine (RILEY et al., 2012; SHEPHARD et al., 2013). AFM_1 and other aflatoxins were also detected in high concentration in the urine of Africans, especially in Ethiopia (TSEGAYE et al., 2016), indicating high health risks to the population of these countries. Three studies in African countries confirmed data on DON excretion by Vidal et al. (2018) showing that the concentration of DON-15-GlcA is greater that the concentration of free DON (ABIA et al., 2013; EZEKIEL et al., 2014; SHEPHARD et al., 2013). Although little frequent in African countries, NIV was detected in relatively high concentrations in positive samples, with the maximum concentration equal to 22,000 ng/L in Cameroon (ABIA et al., 2013). The mycotoxin with the highest concentration reported (241,460 ng/L) was Citrinin in Nigeria (SARKANJ et al., 2018). However, the significance of these levels in terms of the level of dietary exposure and the risk represented to human health in these African countries has to be better elucidated.

Figure 3 - Number of recent studies (2009 to date) on urinary mycotoxin biomarkers per country



Source: Own authorship.

In American countries, all surveys reported the occurrence of single urinary biomarkers, except for the study by Gerding et al. (2015) in Haiti (Table 5). In a robust study, Torres et al. (2014) investigated the presence of FB_1 in 1,236 samples of urine from women in Guatemala, and found 65% of samples containing levels up to 46,600 ng/L. Both studies carried out in Brazil found high incidence of AFM_1 in urine samples, with 65% and 66% positive samples, although at low concentrations, ranging from 0.25 to 29.9 ng/L (JAGER et al., 2016; ROMERO et al., 2010). AFM_1 was also detected in the United States (JOHNSON et al., 2010) and Haiti (SCHWARTZBORD; SEVERE; BROWN, 2017) with maximum concentration equal to 935.49 pg/mg creatinine and 96.5 ng/L, respectively. Taking into account the percentage of AFM_1 excretion reported by Zhu et al. (1987), it may be concluded that there is a potential risk to human health in American countries.

Five Asian countries conducted studies which most of them happened in Bangladesh and China (Table 5). Studies carried out in Bangladesh show high incidence of CIT and DH-CIT, with frequencies ranging from 92 to 97%, and 54 to 97%, respectively (ALI et al., 2015a; GERDING et al., 2015). DON and AFM_1 were detected in 27-31% (ALI et al., 2016c, 2017a) and 42-50% (ALI; BLASZKEWICZ; DEGEN, 2016b) of the samples, respectively. Turner et al. (2011) studied DON and its metabolites in China, and detected DON in 97% urine samples at a maximum concentration of 29,900 ng/L. Deng et al. (2018) confirmed high frequencies of DON in Chinese urine with 93% of positive samples, with maximum level of 47,000 ng/L. These data indicate high level of exposure to DON in food products in China. High levels of AFM_1 (160-550 ng/L) were reported Thailand, but only in 5% of the 60 samples analyzed (WARTH et al., 2014).

DON has been the most reported mycotoxin in European countries, which percentages of positives samples ranging from 8% to 100% (Table 5). The largest concentration was reported in Croatia, where the maximum concentration was 275,000 ng/L in 76% positives samples. Huybrechts et al. (2015) investigated the presence of 32 different mycotoxins (including NIV, FUS-X, CIT, and ZEN and DON metabolites) in 32 urine samples from Belgium. Deoxynivalenol-15-glucuronide was detected in all samples. Heyndrickx et al. (2015) investigated the presence of 28 mycotoxins (including ENs, CIT, FUS-X, and ZEN and DON metabolites) in 239 adult urine and 155 child urine samples in Belgium. They also reported 100% positive samples for DON-15-glucuronide, with concentrations ranging from 1.1 to 460.8 ng/L. DON metabolites were detected at a maximum level of 550,000 ng/L indicating high levels of exposure to DON, which may

be a probable health hazard in Belgium. DON was also detected in 68% samples in Spain, in a range of 2,800 to 69,100 ng/g creatinine (RODRÍGUEZ-CARRASCO et al., 2014).

OTA was the second most studied mycotoxin in European countries, which percentages of positives samples ranging from 10% to 100%. Gerding et al. (2015) detected OTA in 15% urine samples from Germany. Föllmann et al. (2016) described the presence of OTA in 90% urine samples from Germany, and OTα in 50% samples. Ali, Muñoz e Degen (2017) also studies urine samples in Germany, detecting OTA in 100% and OTα in 78% of them, with maximum concentration of 1,820 ng/L and 14,250 ng/L, respectively. OTA was also detected in Belgium (10 to 66%), Croatia (10 to 63%), Italy (100%), Portugal (72.1 to 100%), Spain (12%), Sweden (51%), and Turkey (83%) (AKDEMIR et al., 2010; CORONEL et al., 2011; DOMIJAN et al., 2009; DUARTE et al., 2009, 2010, 2012; EDIAGE et al., 2012; HEYNDRICKX et al., 2015; HUYBRECHTS et al., 2015; KLAPEC et al., 2012; SARKANJ et al., 2013; SOLFRIZZO; GAMBACORTA; VISCONTI, 2014; WALLIN et al., 2015;). Moreover, recent data on the occurrence of multiple mycotoxins in biomarkers of urine in European countries raise a growing concern about the potential impact of combined effects of mycotoxins on human health.

Table 5 - Summary of studies on urinary mycotoxin biomarkers in African, American, Asian and European countries (2009 to date)

continue

Country	Samples analyzed (*N*)	Mycotoxin	Positive samples		LOD (ng/L)	Concentration (ng/L)		Analytical method	Reference
			n	%		Range	Mean		
Africa:									
Cameroon[1]	145 (HIV positive)	AFM_1	15	10	50	< LOQ – 1,380	50	LC-MS/MS	ABIA et al. (2013)
		FB_1	5	3	500	< LOQ – 14,800	630		
		FB_2	1	1	500	< LOQ	-		
		OTA	25	17	50	< LOQ – 1,870	80		
		DON	8	6	4,000	< LOQ	-		
		DON-15-GlcA	62	43	3,000	< LOQ – 96,200	5,490		
		DON-3-GlcA	16	11	6,000	< LOQ – 22,500	3,930		
		ZEN	4	3	400	< LOQ – 1,420	220		
		ZEN-14-GlcA	4	3	1,000	3,380 – 31,000	810		
		α-ZEL	2	1	500	< LOQ	-		
		NIV	20	14	3,000	< LOQ – 22,000	2,610		
	30 (HIV negative)	AFM_1	1	3	50	< LOQ	-		
		FB_1	1	3	500	< LOQ	-		
		FB_2	0	0	500	< LOD	-		
		OTA	3	10	50	< LOQ – 830	60		

Table 5 - Summary of studies on urinary mycotoxin biomarkers in African, American, Asian and European countries (2009 to date)

continue

Country	Samples analyzed (*N*)	Mycotoxin	Positive samples		LOD (ng/L)	Concentration (ng/L)		Analytical method	Reference
			n	%		Range	Mean		
		DON	3	10	4,000	< LOQ	-		
		DON-15-GlcA	10	33	3,000	< LOQ – 46,600	5,620		
		DON-3-GlcA	3	10	6,000	< LOQ – 22,800	4,130		
		ZEN	0	0	400	< LOD	-		
		ZEN-14-GlcA	0	0	1,000	< LOD	-		
		α-ZEL	0	0	500	< LOD	-		
		NIV	5	17	3,000	< LOQ – 20,200	3,220		
Cameroon[2]	220	OTA	70	32	30	40 – 2,400	200	LC-MS	EDIAGE et al. (2013)
		DON	37	17	40	100 – 77,000	2,220		
		AFM_1	31	14	10	60 – 4,700	330		
		FB_1	24	11	10	60 – 48,000	2,960		
		ZEN	9	4	100	650 – 5,000	970		
		β-ZEL	18	8	10	20 – 12,500	1,520		
		α-ZEL	9	4	310	260 – 1,300	980		

Table 5 - Summary of studies on urinary mycotoxin biomarkers in African, American, Asian and European countries (2009 to date)

continue

Country	Samples analyzed (*N*)	Mycotoxin	Positive samples		LOD (ng/L)	Concentration (ng/L)		Analytical method	Reference
			n	%		Range	Mean		
Egypt	98	AFM_1	44	35	2	4.1 – 408.6[17]	19.7[17]	LC-MS	PIEKKOL A et al. (2012)
		DON	63	47	NR	0.5 – 59.9[18]	2.8[18]		
		DOM-1	2	3	200	0.10 – 0.12[18]	0.11[18]		
Ethiopia	349	AFM_1	253	72	NR	4.8 – 4,331[17]	199.8[17]	ELISA	TSEGAYE et al. (2016)
Ethiopia[3]	200	AFB_2	9	4	25	< 50 - 63	47	LC-MS/MS	AYELIGN et al. (2017)
		AFG_1	6	3	25	54 - 65	61		
		AFG_2	5	2	25	66 - 70	68		
		AFM_1	14	7	25	60 - 70	64		
Nigeria[4]	120	DON	1	1	4,000	2,000**	-	LC-MS/MS	EZEKIEL et al. (2014)
		DON-15-GlcA	6	5	NR	< LOD – 209,000	3,500		
		ZEN	1	1	400	300**	-		
		ZEN-14-GlcA	8	7	1,000	< LOD – 44,500	9,500		
		FB_1	16	13	2,000	< LOD – 12,800	4,600		
		FB_2	2	1.7	2,000	< LOD – 1,000**	1,000		
		AFM_1	17	14	50	< LOD – 1,500	300		
		OTA	34	28	50	< LOD – 600	200		

Table 5 - Summary of studies on urinary mycotoxin biomarkers in African, American, Asian and European countries (2009 to date)

continue

Country	Samples analyzed (*N*)	Mycotoxin	Positive samples		LOD (ng/L)	Concentration (ng/L)		Analytical method	Reference
			n	%		Range	Mean		
Nigeria[5]	120	ZEN	98	82	1	30 – 19,990	750	LC-MS/MS	SARKANJ et al. (2018)
		OTA	94	78	0.3	3 - 310	50		
		AFM_1	87	72	0.3	1 - 620	40		
		FB_1	85	71	1	80 – 14,880	1090		
		NIV	40	33	50	240 – 3,020	950		
		DON	23	19	50	80 – 6,220	2370		
		DH-CIT	69	57	3	50 – 16,890	2390		
		AOH	8	7	10	30 – 200	60		
		CIT	79	66	3	15 – 241,460	5960		
		α-ZEL	5	4	3	520 – 2,520	1270		
		β-ZEL	7	6	1	60 – 2,740	880		
South Africa	53	FB_1	51	96	40	40 – 4,940	841	LC-MS/MS	SHEPHARD et al. (2013)
		DON	46	87	450	450 – 53,400	4,940		
		α-ZEL	49	92	9	9 – 3,720	247		
		β-ZEL	40	75	16	16 – 5,940	244		
		ZEN	53	100	2	12 – 3,150	204		

Table 5 - Summary of studies on urinary mycotoxin biomarkers in African, American, Asian and European countries (2009 to date)

continue

Country	Samples analyzed (*N*)	Mycotoxin	Positive samples		LOD (ng/L)	Concentration (ng/L)		Analytical method	Reference
			n	%		Range	Mean		
		OTA	52	98	2	2 – 432	24		
		AFM_1	0	0	1	-	-		
		FB_1	27	51	100	< LOD – 3,200	NR	LC-	
		DON	7	13	4,000	< LOD – 14,000	NR	MS/MS	
		DON-3-GlcA	14	26	6,000	< LOD - <	NR		
		DON-15-GlcA	29	55	3,000	20,000	NR		
		NIV	9	17	1,000	< LOD – 47,000	NR		
						< LOD – 3,700			
Tanzania	147	FB_1	141	96	20	135.4 – 206.7	167.3	LC-MS	SHIRIMA et al. (2013)
Tanzania	166 (First day)	DON	85	51	500	800 – 1,400	NR	LC-MS	SREY et al. (2014)
	166 (After 6 months)	DON	116	70	500	1,700 – 3,200	NR		
	166 (After 12 months)	DON	133	80	500	4,100 – 7,900	NR		

Table 5 - Summary of studies on urinary mycotoxin biomarkers in African, American, Asian and European countries (2009 to date)

continue

Country	Samples analyzed (*N*)	Mycotoxin	Positive samples		LOD (ng/L)	Concentration (ng/L)		Analytical method	Reference
			n	%		Range	Mean		
Tanzania	166 (First day)	FB_1	157	98	20	257.4 – 382.9	313.9	LC-MS	SHIRIMA et al. (2015)
	166 (After 6 months)	FB_1	141	96	20	135.4 – 206.7	167.3		
	166 (After 12 months)	FB_1	146	100	20	464.5 – 698.2	569.5		
Tunisia[6]	110	α-ZAL	31	28	200	700 – 11,800	3,900	LC-MS/MS	BELHASSEN et al. (2015)
		β-ZAL	4	4	300	< LOQ	-		
		ZEN	2	2	30	< LOQ	-		
America:									
Brazil	69	AFM_1	45	65	0.6	1.8 – 39.9	5.96	HPLC	ROMERO et al. (2010)
Brazil[7]	113	AFM_1	74	66	0.075	0.25 – 12.68	1.11	HPLC	JAGER et al. (2016)
Guatemala	1,236 (women)	FB_1	803	65	100	0.0 – 46,600	970	LC-MS	TORRES et al. (2014)

Table 5 - Summary of studies on urinary mycotoxin biomarkers in African, American, Asian and European countries (2009 to date)

continue

Country	Samples analyzed (*N*)	Mycotoxin	Positive samples		LOD (ng/L)	Concentration (ng/L)		Analytical method	Reference
			n	%		Range	Mean		
Haiti[8]	142	DON-GlcA	21	30	1,000	< 4,00 -16,900	3,200	LC-MS/MS	GERDING et al. (2015)
		α-ZEL	3	4	125	520 – 2,490	1,460		
		AFM_1	8	11	25	< 100 – 140	60		
		DH-CIT	14	20	100	< 200 – 4,340	490		
		FB_1	3	4	12.5	230 – 700	440		
		EnB	3	4	1.25	21 – 65	36		
		OTA	33	47	10	< 75 – 225	109		
		DON	17	24	1,000	< 4,000 – 16,900	3,200		
Haiti	52	AFM_1	11	21	2.5	2.94 – 96.5	29.5	HPLC	SCHWARTZBORD et al. (2017)
United States	179	AFM_1	21	12	0.5	1.89 – 935.49[17]	223.85[17]	HPLC	JOHNSON et al. (2010)
United States	30	ZEN	41	NR	NR	< LOD – 310	100	LC-MS/MS	FLECK et al. (2016)
		α-ZEL	10	NR	NR	< LOD - 250	110		
Asia:									
Bangladesh	32 (Rural)	CIT	31	97	20	< LOD – 1,220	140	LC-MS/MS	ALI et al. (2015a)
		DH-CIT	29	91	50	< LOD – 7,470	200		

Table 5 - Summary of studies on urinary mycotoxin biomarkers in African, American, Asian and European countries (2009 to date)

continue

Country	Samples analyzed (*N*)	Mycotoxin	Positive samples		LOD (ng/L)	Concentration (ng/L)		Analytical method	Reference
			n	%		Range	Mean		
	37 (Urban)	CIT	34	92	20	< LOD – 450	60		
		DH-CIT	20	54	50	< LOD – 360	80		
Bangladesh[5]	54	DON	28	52	160	< LOD – 7,160	860	LC-MS/MS	ALI et al. (2015b)
Bangladesh[9]	95	AFM_1	8	8	25	< 100 – 120	60	LC-MS/MS	GERDING et al. (2015)
		DH-CIT	75	71	100	< 200 – 58,820	2,750		
		FB_1	1	1	12.5	-	NR		
		EnB	2	2	1.25	11.9 – 18.7	19		
		OTA	76	72	10	< 75 -2,010	203		
Bangladesh	62 (Summer)	OTA	44	71	10	< LOD – 550	60	LC-MS/MS	ALI et al. (2016a)
		OTα	33	53	20	< LOD – 6,100	210		
		CIT	59	95	20	< LOD – 1,220	100		
		DH-CIT	44	71	50	< LOD – 5,390	420		
	62 (Winter)	OTA	61	98	10	< LOD – 1,750	190		
		OTα	39	62	20	< LOD – 790	90		
		CIT	57	92	20	< LOD – 5,030	590		
		DH-CIT	60	97	50	< LOD – 46,440	3,180		

Table 5 - Summary of studies on urinary mycotoxin biomarkers in African, American, Asian and European countries (2009 to date)

continue

Country	Samples analyzed (*N*)	Mycotoxin	Positive samples		LOD (ng/L)	Concentration (ng/L)		Analytical method	Reference
			n	%		Range	Mean		
Bangladesh[5]	62 (Summer)	DON	17	27	160	< LOD – 1,780	170	LC-MS/MS	ALI et al. (2016b)
	62 (Winter)	DON	19	31	160	< LOD – 1,210	160		
Bangladesh	52 (Rural)	AFM_1	26	50	30	34 – 348	99	ELISA	ALI et al. (2016c)
	43 (Urban)	AFM_1	18	42	30	31 – 84	54		
Bangladesh	62 (Summer)	AFM_1	25	40	1.7	1.7 – 104.0	13.5	HPLC	ALI et al. (2017a)
	62 (Winter)	AFM_1	26	42	1.7	1.8 – 189.9	27.7		
China	77	FB_1	64	83	20	0.01 – 253.61[18]	7.2[18]	LC-MS/MS	XU et al. (2010)
China[5]	60	DON	58	97	NR	< LOD – 29,900	4,800	LC-MS	TURNER et al. (2011)
China	600	AFM_1	510	85	100	LOD – 4,900	NR	ELISA	LEI et al. (2013)

Table 5 - Summary of studies on urinary mycotoxin biomarkers in African, American, Asian and European countries (2009 to date)

continue

Country	Samples analyzed (*N*)	Mycotoxin	Positive samples		LOD (ng/L)	Concentration (ng/L)		Analytical method	Reference
			n	%		Range	Mean		
China	151	DON	140	93	500	< LOD – 47,000	8,250	LC-MS/MS	DENG et al. (2018)
		DOM-1	3	2	100	< LOD - 230	52		
Iran[10]	110	DON + DON-GlcA	79	72	300	< LOD – 6,500	1,300	LC-MS	TURNER et al. (2012)
Iran	70	AFM_1	15	21	NR	0 – 42	1.15	ELISA	MASON et al. (2015)
Malaysia	160	AFM_1	98	61	11	0.0 – 74.7	23.4	ELISA	REDZWAN et al. (2012)
Thailand[11]	60	AFM_1	3	5	50	160 - 550	330	LC-MS/MS	WARTH et al. (2014)
		OTA	1	2	50	< 150	-		
		DON-3-GlcA	3	5	4,000	< 6,000 – 7,000	4,300		
		DON-15-GlcA	7	12	2,000	< 3,000 – 16,800	8,100	HPLC	
Europe:									
Belgium[12]	40	DON	5	13	2,850	3.7 - 67[18]	29.62[18]	LC-MS/MS	EDIAGE et al. (2012)
		OTα	3	7	30	2.5 – 6.0[18]	4.3[18]		
		CIT	1	3	1,880	-	-		
		β-ZEL	4	10	1,100	2.5 - 20[18]	9.45[18]		

Table 5 - Summary of studies on urinary mycotoxin biomarkers in African, American, Asian and European countries (2009 to date)

continue

Country	Samples analyzed (*N*)	Mycotoxin	Positive samples		LOD (ng/L)	Concentration (ng/L)		Analytical method	Reference
			n	%		Range	Mean		
		ZEN	4	10	1,240	< LOQ* – 10.8[18]	NR		
		4-OH OTA	1	3	120	-	-		
		OTA	4	10	30	0.04 – 0.3[18]	0.16[18]		
Belgium[13]	239	CIT	140	59	1	2.2 – 1398.0	56.7	LC-MS/MS	HEYNDRICKX et al. (2015)
	(Adults 19	DH-CIT	14	12***	10	143.1 – 2117.7	752.0		
	- 34 years)	DON	89	37	200	0.5 – 129.8	3.9		
		DON-3-GlcA	184	77	200	0.5 – 126.2	7.5		
		DON-15-GlcA	238	100	200	1.1 – 460.8	53.8		
		DOM-GlcA	53	22	200	0.6 – 172.0	16.9		
		OTA	84	35	1	2.7 – 368.1	27.8		
		α-ZEL	1	< 1	61	-	5.0		
		β-ZEL-14-GlcA	2	1	117	0.6 – 1.0	0.8		
	155	CIT	112	72	1	1.6 – 392.8	31.4		
	(Children 3	DH-CIT	7	6***	10	259.4 – 887.3	550.7		
	- 6 years)	DON	109	70	200	0.5 – 32.5	5.2		
		DON-3-GlcA	141	91	200	0.7 – 43.0	10.6		
		DON-15-GlcA	155	100	200	4.3 – 343.0	58.4		

Table 5 - Summary of studies on urinary mycotoxin biomarkers in African, American, Asian and European countries (2009 to date)

continue

Country	Samples analyzed (*N*)	Mycotoxin	Positive samples		LOD (ng/L)	Concentration (ng/L)		Analytical method	Reference
			n	%		Range	Mean		
		DOM-GlcA	26	17	200	1.1. – 526.1	91.7		
		OTA	79	51	1	3.8 – 3683.0	79.5		
		α-ZEL	0	0	61	-	-		
		β-ZEL-14-GlcA	0	0	117	-	-		
Belgium[14]	32	DON	19	72	200	0 – 3,000	436	LC-MS/MS	HUYBRECHTS et al. (2015)
		DON-3-GlcA	29	91	200	< LOQ – 550,000	10,650		
		DON-15-GlcA	32	100	NR	3,000 – 420,000	82,620		
		DOM-GlcA	8	25	200	800 – 16,400	4,600		
		CIT	19	69	1	2 – 117	26		
		HO-CIT	23	59	10	13 – 210	35		
		OTA	22	66	1	3 – 33	9.5		
Croatia	63 (year 2000)	OTA	24	38	5	5 - 86	13.5	HPLC	DOMIJAN et al. (2009)
	63 (year 2005)	OTA	9	14	5	5 - 15	3		

Table 5 - Summary of studies on urinary mycotoxin biomarkers in African, American, Asian and European countries (2009 to date)

continue

Country	Samples analyzed (*N*)	Mycotoxin	Positive samples		LOD (ng/L)	Concentration (ng/L)		Analytical method	Reference
			n	%		Range	Mean		
Croatia	40	OTA	25	63	16	< LOD – 1,110	NR	HPLC	KLAPEC et al. (2012)
		OTα	40	100	19	110 – 7,570	NR		
Croatia	40	DON	31	76	4,000	< LOD – 275,000	18,300	LC-MS/MS	ŠARKANJ et al. (2013)
		DON-3-GlcA	33	83	6,000	< LOD – 298,100	28,800		
		DON-15-GlcA	39	98	3,000	< LOD – 1237,700	120,400		
		OTA	4	10	50	< LOD – < LOQ	-		
France	76	DON	75	99	50	800 – 28,800	NR	LC-MS/MS	TURNER et al. (2010b)
		DOM-1	26	34	50	200 – 2,800	NR		
Germany[15]	101	DON	30	29	500	< LOD – 17,340	3,380	LC-MS/MS	GERDING, CRAMER AND HUMPF (2014)
		T-2	1	1	250	< 500	-		
		EnB	20	20	5	< 12.5	-		
		DON-3-GlcA	84	82	500	< LOD – 92,950	12,210		
		ZEN-14-GlcA	4	4	750	< 1,500	-		
		DH-CIT	12	12	200	< 500	-		
Germany[16]	50	DON-GlcA	54	27	1,000	< 4,000 – 60,900	11,200	LC-MS/MS	GERDING et al. (2015)
		DH-CIT	28	14	100	< 200 - 330	120		

Table 5 - Summary of studies on urinary mycotoxin biomarkers in African, American, Asian and European countries (2009 to date)

continue

Country	Samples analyzed (*N*)	Mycotoxin	Positive samples		LOD (ng/L)	Concentration (ng/L)		Analytical method	Reference
			n	%		Range	Mean		
		EnB	14	7	1.25	10 - 14	12		
		OTA	30	15	10	<75 - 82	40		
		DON	16	8	1,000	< 4,000	2,000		
Germany	30	DON	30	100	150	850 – 14,634	7,145	LC-MS/MS	FÖLLMANN et al. (2016)
		DOM-1	15	50	100	100 – 228	100		
		CIT	30	100	20	7 – 178	36		
		DH-CIT	30	100	50	6 – 506	136		
		ZEN	30	100	2	4 – 100	36		
		α-ZEL	10	33	10	< LOD – 75	15.5		
		β-ZEL	6	20	10	10 – 37	8		
		OTA	27	90	10	10 – 228	93	HPLC	
		OTα	15	50	10	10 – 310	201		
Germany	50	DON	50	100	160	1,060 – 38,440	9,020	LC-MS/MS	ALI et al. (2016b)
		DOM-1	20	40	100	< LOD - 730	210		
Germany	50	OTA	50	100	10	20 – 1,820	210	HPLC	ALI et al. (2017b)
		OTα	39	78	10	< LOD – 14,250	1,330		

Table 5 - Summary of studies on urinary mycotoxin biomarkers in African, American, Asian and European countries (2009 to date)

continue

Country	Samples analyzed (*N*)	Mycotoxin	Positive samples		LOD (ng/L)	Concentration (ng/L)		Analytical method	Reference
			n	%		Range	Mean		
Italy[5]	52	DON	50	96	1,500[19]	< LOD - 67,360	11,890	LC-MS/MS	SOLFRIZZO, GAMBACORTA AND VISCONTI (2014)
		AFM_1	3	6	20[19]	< LOD – 146	68		
		FB_1	29	56	10[8]	< LOD – 352	55		
		ZEN	52	100	7[19]	NR - 120	57		
		α-ZEL	52	100	30[19]	NR – 176	77		
		β-ZEL	51	98	54[19]	< LOD – 135	90		
		OTA	52	100	6[19]	NR – 2,129	144		
Portugal	43	OTA	31	72	8[19]	< LOD - 71	26	HPLC	DUARTE et al. (2009)
Portugal	155	OTA	143	92	8[19]	< LOD – 69	18	HPLC	DUARTE et al. (2010)
Portugal	95 (Summer)	OTA	77	81	2.4	< LOD – 40	16	HPLC	DUARTE et al. (2012)
	95 (Winter)	OTA	83	87	2.4	< LOD – 71	22		
Spain	72	OTA	9	13	34	57 – 562	237	HPLC	CORONEL et al. (2011)
		OTα	43	61	23	56 – 2,894	441		

Table 5 - Summary of studies on urinary mycotoxin biomarkers in African, American, Asian and European countries (2009 to date)

continue

Country	Samples analyzed (*N*)	Mycotoxin	Positive samples		LOD (ng/L)	Concentration (ng/L)		Analytical method	Reference
			n	%		Range	Mean		
Spain	54	DON	37	68	120	2,800 – 69,100[18]	23,300[18]	GC-MS/MS	RODRÍGUEZ-CARRASCO et al. (2014)
		DOM-1	2	4	250	< LOD -4,200[18]			
		NIV	7	13	500	< LOD -17,600[18]			
		HT-2	4	7	2,000	< LOD -15,800[18]			
Sweden	326	DON	292	90	500[19]	< LOD – 65,800	NR	LC-MS	WALLIN et al. (2013)
Sweden	252	DON	158	63	NR	NR	5,380	LC-MS/MS	WALLIN et al. (2015)
		DOM-1	20	8	NR	NR	2,320		
		ZEN	92	37	NR	NR	90		
		α-ZEL	53	21	NR	NR	130		
		β-ZEL	45	18	NR	NR	100		
		FB_1	15	6	NR	NR	70		
		FB_2	57	23	NR	NR	60		
		OTA	128	51	NR	NR	900		
		NIV	42	17	NR	NR	130		

Table 5 - Summary of studies on urinary mycotoxin biomarkers in African, American, Asian and European countries (2009 to date)

conclusion

Country	Samples analyzed (*N*)	Mycotoxin	Positive samples		LOD	Concentration (ng/L)		Analytical method	Reference
			n	%	(ng/L)	Range	Mean		
Turkey	233	OTA	193	83	6	< LOD -76.60[18]	14.34[18]	HPLC	AKDEMIR et al. (2010)
United Kingdom[5]	62 (Adults 18 -64 years)	DON	51	82	120	NR	3.02[18]	LC-MS	WELLS et al. (2017)
		DON-GlcA	51	82	250	NR	16.12[18]		
	64 (Vegetarians)	DON	64	100	120	NR	5.06[18]		
		DON-GlcA	64	100	250	NR	21.18[18]		
United Kingdom[5]	80 (Children 3 -9 years)	DON	NR	NR	250[19]	< LOD – 55.2	8.4	LC-MS	PAPAGEORGIOU et al. (2018a)
		DON-DlcA	NR	NR	500[19]	3.8 – 182.0	33.4		
	78 (Adolescents 10 -17 years)	DON	NR	NR	250[19]	< LOD – 17.0	3.6		
		DON-GlcA	NR	NR	500[19]	< LOD – 78.6	17.3		
United Kingdom[5]	40 (Elderly)	DON	29	72.5	250[19]	< LOD – 42,000	2,250	LC-MS	PAPAGEORGIOU et al. (2018b)
		DON-GlcA	36	90	NR	< LOD - 144,000	13,650		
	62 (Adults)	DON	58	93.5	250[19]		2,350		
		DON-GlcA	62	100	NR	100 – 13,800	12,100		
						700 – 45,000			

[1]Mycotoxins analysed with 0% positive samples: DOM-1, β-ZEL, T-2 and HT-2.

[2]Mycotoxins analysed with 0% positive samples: AFB_1, CIT, T-2, H-T2, HFB_1, DOM-1, AFB_1-N^7-Gua, OTα, 4-OH OTA, DON-3Glu and ZEN-Glu.

[3]Mycotoxins analysed with 0% positive samples: AFB_1.

[4]Mycotoxins analysed with 0% positive samples: DON-3-GlcA, DOM-1, NIV, α-ZEL, β-ZEL, T-2 and HT-2.
[5]Mycotoxins analysed with 0% positive samples: DOM-1.
[6]Mycotoxins analysed with 0% positive samples: ZAN, α-ZEL and β-ZEL.
[7]Mycotoxins analysed with 0% positive samples: AFB_1-N^7-guanine.
[8]Mycotoxins analysed with 0% positive samples: ZAN-14-GlcA, ZAN, ZEN, α-ZEL-14-GlcA, ZEN-14-GclA, OTα, β-ZEL, β-ZEL-14-GlcA, AFB_1, AFB_2, AFG_1, AFG_2, T-2, HT2 and HT2-4-GlcA.
[9]Mycotoxins analysed with 0% positive samples: DON-GlcA, ZAN-14-GlcA, ZAN, ZEN, α-ZEL, α-ZEL-14-GlcA, ZEN-14-GclA, OTα, β-ZEL, β-ZEL-14-GlcA, AFB_1, AFB_2, AFG_1, AFG_2, T-2, HT-2, HT-2-4-GlcA and DON.
[10]Mycotoxins analysed with 0% positive samples: DOM-1 and FB_1.
[11]Mycotoxins analysed with 0% positive samples: FB_1, FB_2, DON, DOM-1, NIV, T-2, HT-2, ZEN, ZEN-14-GlcA, α-ZEL and β-ZEL.
[12]Mycotoxins analysed with 0% positive samples: DON-3-GlcA, DOM, AFB_1-N^7-Gua, AFM_1, AFB_1, HFB_1, HT-2, FB_1, T-2, α-ZEL and ZEN-14-GlcA.
[13]Mycotoxins analysed with 0% positive samples: AFB_1, AFB_2, AFG_1, AFG_2, AFM_1, FB_1, FB_2, FB_3, HFB_1, OTα, FUS-X, DAS, DOM-1, 3ADON, 3ADON15GlcA, 15ADON, 15ADON3GlcA, β-ZEL, α-ZEL-7-GlcA and α-ZEL-14-GlcA.
[14]Mycotoxins analysed with 0% positive samples: 3-AcDON-15-GlcA, DOM-1, 15-AcDON-3-GlcA, FUS-X, 15-AcDON, 3-AcDON, β-ZEL-14-GlcA, OTα, AFG_2, AFM_1, AFG_1, AFB_2, AFB_1, DAS, α-ZEL-14-GlcA, ZEN-14-GlcA, HT-2, α-ZEL-7-GlcA, β-ZEL, FB_1, T-2, α-ZEL, ZEN, FB_3 and FB_2.
[15]Mycotoxins analysed with 0% positive samples: HT-2, HT-2-4-GlcA, FB_1, FB_2, AFB_1, AFB_2, AFG_2, AFM_1, ZAN, ZEN, α-ZEL, β-ZEL, ZAN-14-GlcA, α-ZEL-14-GlcA, β-ZEL-14-GlcA, OTA and OTα.
[16]Mycotoxins analysed with 0% positive samples: ZAN-14-GlcA, ZAN, ZEN, α-ZEL, α-ZEL-14-GlcA, ZEN-14-GclA, OTα, β-ZEL, β-ZEL-14-GlcA, AFB_1, AFB_2, AFG_1, AFG_2, AFM_1, T-2, HT2, HT2-4-GlcA, FB_1
[17] result shown in pg/mg creatinine.
[18] results shown in ng/mg creatinine.
[19]values were reported as LOQ.
*LOQ = 2,480 ng/L.
**Values were reported as half LOQ (LOQ/2).
*** Not all samples were analysed for DH-CIT (Adults: 120; Children:124).
NR – Not reported. LOD – Limit of detection. LOQ - Limit of quantification. LC-MS – Liquid chromatography coupled to mass spectrometry. LC-MS/MS – Liquid chromatography coupled to tandem-mass spectrometry. ELISA - Enzyme Linked ImmunoSorbent Assay.
DON - deoxynivalenol. 15-ADON - 15-acetyl-deoxynivalenol. 3-ADON - 3-acetyl-deoxynivalenol. T-2 - T-2-toxin. HT-2 - HT-2-toxin. FUM – Fumonisins. FB_1 - Fumonisin B_1. FB_2 - Fumonisin B_2. FB_3 - Fumonisin B_3. AFB_1 – aflatoxin B_1. AFB_2 – Aflatoxin B_2. AFG_1 - Aflatoxin G_1. AFG_2 - Aflatoxin G_2. AFM_1 - Aflatoxin M_1. AFB_1-N^7-guanine - Aflatoxin B_1-N (7)-guanine. ZEN - Zearalenone. ZAN - Zearalanone. α-ZEL - α-Zearalenol. β-ZEL - β-Zearalenol. α-ZAL - α-Zearalanol. β-ZAL - β-Zearalanol. ZEN-14-GlcA - Zearalenone-14-O-glucuronide. ZAN-14-GlcA - Zearalanone-14-O-glucuronide. α-ZEL-14-GlcA - α-zearalenol-14-O-glucuronide. β-ZEL-14-GlcA - β-zearalenol-14-O-glucuronide. OTA - Ochratoxin A. OTα - Ochratoxin alpha. FUS-X – Fusarenon X. DAS – Diacetoxyscirpenol. NEO – Neosolaniol. STER – Sterigmatocystin. ENA – Enniatin A. ENA_1 – Enniatin A_1. ENB - Enniatin B. ENB_1 - Enniatin B_1. EnB_4 – Enniatin B_4. BEA – Beauvericin. DH-CIT – Dihydrocitrinone. EnB – Enniatin B. DOM-1 -Deepoxy-deoxynivalenol. DH-CIT -Dihydrocitrinone. AOH – Alternariol. CIT – Citrinin. HFB_1 - Hydrolyzed FB_1. 4-OH OTA – 4 Hydroxy OTA. DON-3Glu - Deoxynivalenol 3-glucoside. ZEN-Glu - Zearalenone glucoside. DON-3-GlcA - Deoxynivalenol-3-glucuronide. DON-15-GlcA - Deoxynivalenol-15-glucuronide. HT-2-4-GlcA - HT-2-toxin 4-O-glucuronide. 3ADON15GlcA - 3-acetyldeoxynivalenol-15- glucuronide. 15ADON3GlcA - 15-acetyldeoxynivalenol-3-glucuronide. DON-GlcA - Deoxynivalenol-glucuronide.

2.5 Concluding remarks

Data on the occurrence of mycotoxin biomarkers in human urine indicated high rates of dietary exposure to AF, FB, ZEN, and DON, especially in African and Asian countries. Although AFM_1, OTA, FB_1, and ZEN and DON metabolites were very frequently reported in human urine samples worldwide, considerable effort should be made in the evaluation of urinary biomarkers in areas where the risk of mycotoxin contamination of staple foods is high, in order to assess actual levels of exposure to dietary mycotoxins. Biomonitoring of multiple biomarkers in urine opens new perspectives for the assessment of dietary exposure to co-occuring mycotoxins in human populations, and for the evaluation of the protective effects of regulations for mycotoxins in food commodities. However, further toxicokinetic studies are needed to provide data on absorption, distribution, metabolization and excretion in humans considering possible mycotoxin mixtures in the diet.

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3 Chapter II: **Performance and application of a "dilute-and- shoot" LC-MS/MS method for determination of mycotoxins in food products in São Paulo, Brazil.**

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Abstract

Mycotoxins are secondary metabolites produced by several species of fungi that occurs in agricultural commodities, which cause a great variety of toxic effects in vertebrates, including humans. In this study, a "dilute-and-shoot" method using ultra-performance liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) was evaluated and applied to assess the incidence of aflatoxins (AF) B_1, B_2, G_1 and G_2, fumonisins (FB) B_1 and B_2, ochratoxin A (OTA), zearalenone (ZEN) and deoxynivalenol (DON) in samples of corn meal (*N* = 4), corn flour (*N*=2), wheat flour (*N*=4), rice (*N*=14) and bean (*N*=12) collected in small-scale farms from São Paulo, Brazil. The performance parameters (apparent recovery, matrix effect, extraction recovery, linearity, limit of quantification and limit of detection) were satisfactory, and the most frequent mycotoxin detected in food samples was FB (7.11-316.04 μg/kg) below the Brazilian regulations. DON was detected in all samples of wheat flour (162.49-324.66 μg/kg) along with FB in 3 samples, and in one sample of bean (46.52 μg/kg). ZEN was detected in 2 samples of rice (4.90-6.78 μg/kg). Results indicated low incidences of mycotoxin in the products evaluated, although the co-occurrence of FB and DON warrants concern about their incidence in wheat flour in Brazil.

Keywords: Aflatoxins. Ochratoxin A. *Fusarium* toxins. Occurrence. LC-MS/MS. Analysis.

3.1 Introduction

Mycotoxins are secondary metabolites produced by fungi species that develop naturally in food products, which cause a great variety of toxic effects in vertebrates, including humans (BRÄSE et al., 2013). Exposure to toxins occurs predominantly by the ingestion of contaminated food, especially cereals and grains, such as corn, wheat, and peanut, among others (OLIVEIRA et al., 2014). The most common toxigenic fungi found in cereals in Brazil include species from the genera *Aspergillus* and *Fusarium* (BORDIN et al., 2015; JAGER et al., 2013; TRALAMAZZA et al., 2016).

The aflatoxins are produced by *Aspergillus* species, mainly *A. flavus*, *A. parasiticus* and *A. nomius* (OLIVEIRA et al., 2014). The main aflatoxin compounds are B_1 (AFB_1), B_2 (AFB_2), G_1 (AFG_1) and G_2 (AFG_2), although AFB_1 has the higher teratogenicity, mutagenic and carcinogenic effects (JAGER et al., 2013). In Brazil, *A. ochraceus* (formerly *A. alutaceus*) is the main producing species of ochratoxin A (OTA), a toxin that interferes with the synthesis of macromolecules in the cells of the renal parenchyma, including DNA, RNA

and proteins (BRÄSE et al., 2013). The *Fusarium* toxins are mycotoxins produced by several species in the genus, including the fumonisins, trichothecenes, and zearalenone (ZEN), among others (BRYDEN, 2012).

Fumonisins are produced mainly by *Fusarium verticillioides*, being fumonisins B_1 (FB_1) and B_2 (FB_2) the most prevalent in natural conditions, although FB_1 is the most toxic compound (FERRIGO; RAIOLA; CAUSIN, 2016). FB_1 has been associated with animal diseases such as equine leucoencephalomalacia and porcine pulmonary edema (OLIVEIRA et al., 2014). ZEN is an estrogenic substance derived from resorcylate acid, produced by several *Fusarium* species, e.g., *F. roseum (F. graminearum)*, *F. culmorum* and *F. equisetum*, among others (OLIVEIRA, 2016). Deoxynivalenol (DON), also called vomitoxin, is a class B trichothecene mainly produced by *F. roseum*, which provokes nausea, diarrhea, reduced nutritional efficiency, gastrointestinal tract injuries and weight loss in animals (WU et al., 2015).

Taking into account the risks posed by mycotoxins to the human health, several countries have determined tolerance limits for these toxins in food products. Brazilian regulations for mycotoxins were revised in 2011, with the introduction of maximum permitted levels (MPL) for aflatoxins (sum of AFB_1, AFB_2, AFG_1 and AFG_2), OTA, fumonisins (sum of FB_1 and FB_2), ZEN and DON in several food products (ANVISA, 2011). Since the adoption of action levels for mycotoxins, a few reports in Brazil indicated high incidence rates (up to 100%) of fumonisins in corn products (BORDIN et al, 2015). In another study (JAGER et al., 2013), corn products showed 42% positive samples for aflatoxins at levels ranging from 0.05 to 8.3 μg/kg (sum of AFB_1, AFB_2, AFG_1 and AFG_2). Bean kernels had the highest incidence (75% positive samples), but at low levels (0.025 to 0.042 μg/kg, sum of all fractions) (JAGER et al., 2013). In Paraná State, DON was detected in 66% of wheat grains samples, with a mean level of 1,895 μg/kg (SANTOS et al., 2013). The data presented in those studies confirm that individual mycotoxins have been frequently detected in Brazilian cereals. Importantly, two or more mycotoxins may be present in the food as a consequence of multiple fungi contamination, or when one species produces more than one type of mycotoxin (e.g., *Fusarium* species) (OLIVEIRA, 2016). However, there is little information on the simultaneous occurrence of mycotoxins in food products in Brazil.

In the past decade, analytical methods based on the “dilute-and-shoot” approach and liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) have been developed for the simultaneous analysis of mycotoxins in foods (SULYOK; KRSKA; SCHUHMACHER, 2007). In the present study, the performance of a “dilute-and-shoot”

LC-MS/MS analytical method was evaluated, aiming to determine the levels of aflatoxins, OTA, fumonisins, ZEN and DON in food products susceptible to mycotoxin contamination in the state of São Paulo, Brazil.

3.2 Material and Methods

3.2.1 Mycotoxin Standards

The standards of AFB_1, AFB_2, AFG_1, AFG_2, OTA, FB_1, FB_2, ZEN and DON were purchased from Sigma-Aldrich (St Louis, MO, USA). Isotopically labeled standards [$13C_{17}$]-AFB_1 (Sigma-Aldrich, St. Louis, MO, USA), [$13C_{20}$]-OTA, [$13C_{34}$]-FB_1, [$13C_{18}$]-ZEN and [$13C_{15}$]-DON (Biopure, Romer Labs, Tulln, Austria) were used as internal standards. Two individual mixtures (10 μg/mL) composed of labeled and unlabeled mycotoxins were prepared in water/acetonitrile (1:1, v/v) which was used to prepare to prepare a standard working solution containing AFB_1, AFB_2, AFG_2, AFG_2, OTA, ZEN, FB_1, FB_2 at 100 ng/mL and DON at 750 ng.mL-1 and a second mixture of the labeled standards were diluted at concentrations of 5 ng/mL for [$13C_{17}$]-AFB_1, [$13C_{20}$]-OTA, [$13C_{34}$]-FB_1, [$13C_{18}$]-ZEN and 100 ng/mL for [$13C_{15}$]-DON. All solutions were kept in freezer at -20ºC. Before use, samples were kept in the dark until room temperature was reached.

For the preparation of all curves, 80 μL of solutions of each calibration point and 20 μL of the labeled standard working solution were added into amber vials provided with glass inserts so that the final concentrations of the internal standards in the vials were of 1 ng/mL of [$13C_{17}$]-AFB_1, [$13C_{20}$]-OTA, [$13C_{34}$]-FB_1, 50 ng/mL of [$13C_{18}$]-ZEN and 100 ng/mL of [$13C_{15}$]-DON in water/acetonitrile (9:1).

3.2.2 Evaluation of Method Performance

The analytical method was evaluated in terms of aparent recovery (R_A), matrix effects (SSE) and extraction recovery (R_E). Additionaly, linearity, limit of quantification (LOQ) and limit of detection (LOD) were also evaluated. RA, SSE and RE parameters were calculated according to the equations (Eq.1), (Eq.2) and (Eq.3), respectively (DI GREGORIO et al., 2017).

$$R_A\ (\%) = 100 \times slope_{spiked\ sample} / slope_{liquid\ standard} \quad \text{(Eq.1)}$$

$$SSE\ (\%) = 100 \times slope_{spiked\ extract} / slope_{liquid\ standard} \quad \text{(Eq.2)}$$

$$R_E\ (\%) = 100 \times R_A / SSE \quad \text{(Eq.3)}$$

Recovery assays were performed for each type of food product susceptible to mycotoxin contamination and regulated in Brazil (BRYDEN, 2012). Triplicate blank samples (1.0 g) of finelly milled corn meal, corn flour, wheat flour, rice and bean were spyked at six concentration levels of the unlabeled mycotoxins. For the 3 highest points, 160 µL, 120 µL and 80 µL of the working solution containing the mixture of mycotoxin standards (prepared as decribed in item 3.2.1) were added to fortify the samples. For the 4 lowest points, the working solution was diluted (1:4), and volumes of 80 µL, 40 µL and 8 µL were added. This resulted in the concentrations of 4.0, 3.0, 2.0, 1.0, 0.5, 0.25 and 0.05 ng/mL for AFB_1, AFB_2, AFG_2, AFG_2, OTA, ZEA, FB_1, FB_2 and 30.0, 22.5, 15.0, 7.5, 3.75, 1.87 and 0.37 ng/mL for DON. The samples were vortexed and conditioned in the dark at room temperature and uncapped for 12 h to allow solvent evaporation and absorption of the analytes by the food matrix. Next, the samples were shaken 2 times for 30 min. in a horizontal shaker (Tecnal, Piracicaba, Brazil) to homogenate the samples.

3.2.3 Sample Preparation

Extraction procedures were performed using a "dilute-and-shoot" approach as described by Sulyok, krska e Schuhmacher (2007), with minor modifications. One gram of milled sample of each food product was weighed in 15-mL Falcon Tubes in duplicate, and 4 mL of acetonitrile:water:acetic acid (80:20:0.1) was added to the tubes. After vortexing for 1 min, the mixtures were shaken in a horizontal shaker (Tecnal, Piracicaba, Brazil) for 60 min. Next, the tubes were vortexed and shaken again for 30 min. The samples were then centrifuged (Quimis, Brazil) at 1,700 g for 5 min. The supernatant was filtered in PTFE 0.22 µm membrane filters (Millex, Millipore Corp.). An aliquot of 80 µL of these solutions were transferred to glass inserts inside vials and mixed with 20 µL of the internal standards working solution (prepared as described in item 3.2.1). The final extracts were vortexed and reserved for LC-MS/MS determinations.

3.2.4 Liquid Chromatographic and Mass Spectrometry conditions

The final extracts were injected into a Waters Acquity I-Class Ultra-Performance LC system (Waters, Milford, MA, USA) equipped with a BEH C_{18} column (2.1 × 50 mm, 1.7 µm) and coupled to a Xevo TQ-S mass spectrometer (Waters, Milford, MA, USA). The column was kept at 40°C during the analyses, and samples were maintained at 15°C. Ten microliters of extracted samples and standards were injected. Elution gradient was accomplished in a mobile phase composed by water (eluent A) and acetonitrile (eluent B),

both containing 0.1% of acetic acid and ammonium acetate 5 mM. After an initial period of 0.5 min at 98% eluent A, the percentage of eluent B was linearly raised to 30% over 4.5 min (5.0 min). Then, eluent B was increased to 96% over 2.0 min, followed by a hold time of 0.5 min (7.5 min). After that, the percentage of eluent B was reduced to 2% over 2 min (8.0 min), and the column re-equilibrated to the initial conditions for 2.0 min. Total chromatographic run time was 10 min, and the mobile phase flow rate was maintained at 0.6 mL/min.

The mass spectrometer was operated in MRM mode using electrospray ionization in positive and negative ion mode, with a capillary voltage of of 3.00 kV for the positive mode and 2.00 kV for the negative, source temperature of 150°C, and desolvation temperature of 500°C. Desolvation gas flow and cone gas flow were maintained at 800 L/h and 150 L/h, respectively. Cone voltage, collision energy, and MRM transitions (major precursor ion > fragment ion) were manually optimized. Quantification and confirmatory MRM transitions for mycotoxins and corresponding optimal mass spectrometric parameters used in the LC-MS/MS analysis in food products are presented in Table 6. Data collection and processing was performed using software MassLynx version 4.1.

In order to evaluate the matrix effects of each food product, individual blank samples of corn meal, corn flour, wheat flour, rice and beans were extracted exactly as described in item 3.2.3. The resulting raw blank extracts were used to prepare matrix-matched standard curves for each product, which were generated by 7 concentration points in triplicates. SSE parameters were stablished by comparing the matrix calibration curve with the corresponding curve prepared in solvent (water/acetonitrile 9:1). Eighty μL of the spiked raw extracts were combined with 20 μL of the IS working solutions for injection into the LC-MS/MS system. By using this procedure, the following mycotoxin levels were achieved in each extract: 4.0, 3.0, 2.0, 1.0, 0.5, 0.25, 0.05 ng/mL for AFB_1, AFB_2, AFG_2, AFG_2, OTA, ZEA, FB_1, FB_2, and 30.0, 22.5, 15.0, 7.5, 3.75, 1.87, and 0.37 ng/mL for DON.

LOD and LOQ values were calculated from the matrix matched calibration curves using signal to noise ratios (S/N) of minimum 3 and 10 for each qualifier MRM transition, respectively. The method characteristics were determined using internal and external calibrations, aiming to verify the efficiency of internal standards to compensate the matrix effects (MARTINS et al., 2018). To quantify the mycotoxins, the ratio of sample peak area to internal standard peak area was calculated and the final concentration was determined through the corresponding calibration line.

Table 6 - Mass spectrometry conditions used for mycotoxin analyses in food products

Mycotoxin	Mass (g/mol)	Molecular ion	Transition (*m/z*)	Cone voltage (V)	Collision energy (V)
Aflatoxin B_1	312.28	$[M+H]^+$	312.7 > 284.9 [a]	94	36
			312.7 > 241.1 [b]	94	22
$[^{13}C_{17}]$-Aflatoxin B_1	329.15	$[M+H]^+$	330.3 > 301.5	94	20
Aflatoxin B_2	314.29	$[M+H]^+$	314.7 > 259.0 [a]	2	28
			314.7 > 287.0 [b]	2	26
Aflatoxin G_1	328.27	$[M+H]^+$	328.9 > 243.0 [a]	2	26
			328.9 > 199.5 [b]	2	38
Aflatoxin G_2	330.29	$[M+H]^+$	330.9 > 245.0 [a]	56	28
			330.9 > 188.9 [b]	56	40
Ochratoxin A	403.08	$[M+H]^+$	404.0 > 238.9 [a]	35	22
			404.0 > 357.9 [b]	35	12
$[^{13}C_{20}]$-Ochratoxin A	423.67	$[M+H]^+$	424.2 > 250.0	30	20
Fumonisin B_1	721.84	$[M+H]^+$	722.5 > 334.0 [a]	50	40
			722.5 > 352.1 [b]	50	30
$[^{13}C_{34}]$-Fumonisin B_1	755.58	$[M+H]^+$	756.6 > 374.4	50	30
Fumonin B_2	70.83	$[M+H]^+$	706.5 > 336.2 [a]	50	35
			706.5 > 318.3 [b]	50	40
Zearalenone	318.15	$[M-H]^-$	317.1 > 175.1 [a]	50	23
			317.1 > 130.9 [b]	50	33
$[^{13}C_{18}]$-Zearalenone	336.23	$[M-H]^-$	335.1 > 185.1	50	31
Deoxinyvalenol	296.32	$[M+H]^+$	397.3 > 249.1 [a]	6	10
			397.3 > 231.1 [b]	6	14
$[^{13}C_{15}]$-Deoxynivalenol	311.21	$[M+H]^+$	312.1 > 98.7	30	35

Source: Own authorship. [a] Transitions used for quantification; [b] Transitions used for confirmation.

3.2.5 Sampling Procedures and Application of the Method

Sampling procedures for collection of food products were performed in small-scale farms from the surroundings of Pirassununga and Descalvado, located in the State of São Paulo, Brazil. Samples of corn meal (N = 4), corn flour (N = 2), wheat flour (N = 4), rice (N = 14) and bean (N = 12) availabe and stored in the households of each farm were collected in June 2016. All food products were commercially available, and had been

purchased by the farmers and stored in the households prior to sample collection. Samples were packed in polyethylene bags and sent immediately to the laboratory, where they were homogenized, finely milled and submitted to analysis for mycotoxins by using the same analytical method as described in items 3.2.3 and 3.2.4.

3.3 Results and Discussion

The method performance parameters (concentration range, LOQ, LOD, R_A using external or internal calibration, SSE and R_E) determined in the food products are presented in Table 7. Overall, the calculated LOQ values for individual mycotoxins varied between 0.8 to 18.8 μg/kg, which were much lower than the MPLs for mycotoxins in foods established by the Brazilian regulations (ANVISA, 2011). These results confirm that the method evaluated is suitable for routine determination of all target mycotoxins in samples of corn meal, corn flour, wheat flour, rice and bean.

Table 7 - Method performance parameters determined in food products[a]

	Conc. Range (μg/kg)	LOQ (μg/kg)	LOD (μg/kg)	R_{Aext}	R_{Aint}	SSE_{int}	R_E
AFB_1	1.25-20	0.8-1.0	0.4-0.5	133-141	101-108	92-99	109-110
AFB_2	1.25-20	0.8-1.0	0.4-0.5	90-112	96-106	93-103	102-107
AFG_1	1.25-20	0.8-1.0	0.4-0.5	61-93	89-109	95-107	94-102
AFG_2	1.25-25	0.9-1.0	0.4-0.6	57-80	92-103	95-97	94-108
OTA	1.25-20	1.0-1.1	0.5-0.7	86-143	97-106	92-99	102-109
FB_1	2.5-25	2.5-2.8	0.9-1.0	153-231	99-109	95-102	97-111
FB_2	2.5-25	2.0-2.5	0.7-0.9	148-211	91-101	90-99	96-102
DON	18.7-150	18.5-18.8	5.8-6.1	33-60	93-103	89-109	93-105
ZEN	0.25-20	0.3-0.6	0.12-0.23	42-72	97-105	95-105	98-107

Source: Own authorship. [a] Expressed as minimum-maximum values; LOQ: Limit of quantification; LOD: Limit of detection; R_A: Apparent recovery; SSE: Matrix effects; R_E: Extraction recovery; AF: aflatoxin; FB: fumonisin; OTA; ochratoxin A; ZEN: zearalenone; DON: deoxynivalenol.

The use of external and internal calibrations was efficient for compensating the matrix effects on the method performance. The overall values obtained for the apparent recovery using external calibration (R_{Aext}) varied markedly among the different mycotoxins and food products, ranging from 33% to 143% (Table 7). However, the addition of internal standards efficiently compensated the matrix effect, since the overall R_{Aint} values ranged from 92 to

109%. The recovery of the extraction procedure (R_E) was calculated by the ratio between the R_{Aint} values obtained through the fortified matrices before the extraction, and the SSE_{int} values obtained through the fortified matrices after extraction, both using internal calibration. As shown in Table 7, recovery values were adequate for the target mycotoxins in all evaluated matrices (93-111%). Finally, the calculated linearity (r^2) of the analytical curves was > 0.99. The satisfactory performance parameters obtained for the analytical method enable its application for the simultaneous determination of mycotoxins in the food products analyzed.

Table 8 presents the incidence and levels (range and mean values) of aflatoxins, OTA, fumonisins, ZEA and DON found in samples of corn meal, corn flour, wheat flour, rice and bean collected in small-scale farms from the state of São Paulo, Brazil. MPL values adopted in Brazil for aflatoxins, OTA, fumonisins, ZEA and DON are also presented in Table 8. Aflatoxins or OTA were not detected in any sample, and although fumonisins, ZEA and DON were quantified in several samples, none exceeded the MPL values adopted in Brazil (ANVISA, 2011).

Fumonisins (FB_1 and FB_2) had the highest incidence in the food products, being detected in 100% samples of corn meal and corn flour at mean levels of 103.06 ± 29.43 μg/kg and 27.92 ± 29.44 μg/kg, respectively (sum of FB_1 and FB_2). These results are consistent with those reported in a previous survey conducted in São Paulo state (BORDIN et al., 2015), in which FB_1 was found in 60% samples of corn-based products at a mean level of 360.4 μg/kg. However, the fumonisin concentrations found in corn meal in the present work were lower than those described previously (mean level of FB_1: 422.9 μg/kg) (BORDIN et al., 2015). Although the number of samples analyzed was low, our results may indicate that manufacturers of corn products have improved their control practices regarding fumonisin contamination.

FB_1 and FB_2 were also detected in 75% of samples of wheat flour, at a mean concentration of 109.19 ± 179.23 μg/kg (sum of fractions), and all samples of this food product had DON (mean level: 244.54 ± 90.55 μg/kg), as shown in Table 8. The high incidence of FB_1 and FB_2 in wheat flour was surprising, since there is no MPL established for fumonisins in this product in Brazil. Accordingly, the maximum level of fumonisins found in wheat flour (316.04 μg/kg) was higher than the highest level determined in corn meal (131.92 μg/kg). Importantly, the co-occurrence of fumonisins and DON was observed in 3 samples (75%) of wheat flour (data not shown), which warrants concern about the incidence of those toxins in wheat flour in Brazil.

Table 8 - Mycotoxin levels in food products from small-scale farms in the state of São Paulo, Brazil

Mycotoxin	*n* (%)	Variation (μg/kg)	Mean ± SD (μg/kg)	MPL (μg/kg)
Corn meal (*N* = 4)				
Aflatoxins	0 (0)	-	-	20
Ochratoxin A	0 (0)	-	-	10
Fumonisins	4 (100)	69.44 - 131.92	103.06 ± 29.43[a]	1500
Zearalenone	0 (0)	-	-	150
Deoxynivalenol	0 (0)	-	-	750
Corn flour (*N* = 2)				
Aflatoxins	0 (0)	-	-	20
Ochratoxin A	0 (0)	-	-	10
Fumonisins	2 (100)	7.11 - 48.74	27.92 ± 29.44[a]	1500
Zearalenone	0 (0)	-	-	150
Deoxynivalenol	0 (0)	-	-	750
Wheat flour (*N* = 4)				
Aflatoxins	0 (0)	-	-	5
Ochratoxin A	0 (0)	-	-	10
Fumonisins	3 (75)	11.54 - 316.04	109.19 ± 179.23[a]	NRE
Zearalenone	0 (0)	-	-	100
Deoxynivalenol	4 (100)	162.49-324.66	244.54 ± 90.55	750
Rice (*N* = 14)				
Aflatoxins	0 (0)	-	-	5
Ochratoxin A	0 (0)	-	-	10
Fumonisins	0 (0)	-	-	NRE
Zearalenone	2 (14)	4.90 - 6.78	5.84 ± 1.33	100
Deoxynivalenol	0 (0)	-	-	750
Bean (*N* = 12)				
Aflatoxins	0 (0)	-	-	5
Ochratoxin A	0 (0)	-	-	10
Fumonisins	0 (0)	-	-	NRE
Zearalenone	0 (0)	-	-	NRE
Deoxynivalenol	1 (8)	-	46.52 ± 0	NRE

Source: Own authorship. *n*: Samples showing concentrations above the limit of determination (LOD) – refer to Table 7 for LOD values; MPL: Maximum permitted level adopted by Brazilian regulation [9]; NRE: No regulation established; [a] Sum of fumonisins B_1 and B_2.

The simultaneous occurrence of *Fusarium* toxins in wheat flour has been described elsewhere. DON, ZEN and other *Fusarium* toxins (e.g., nivalenol, 3-acetyldeoxynivalenol and 15-acetyldeoxynivalenol) were detected in the majority of 348 samples of wheat flour

marketed in Hebei province of China, although DON was the most prevalent, being detected in 91.4% of samples analyzed (LIU et al., 2015). The mean concentration reported for DON (240 μg/kg) (LIU et al., 2015) was similar to the level described in the present study. In a recent survey conducted in the Brazilian states of São Paulo, Paraná and Rio Grande do Sul Brazil (DUFFECK et al., 2017), ZEN and DON were found in 39% and 55% of wheat grain samples, respectively, with overall mean levels of 79.78 μg/kg and 795.2 μg/kg, respectively, which is much high than the values reported in our work. In the present study, DON was also detected in one sample (8.3%) of bean, as shown in Table 8. Although the concentration found was low (46.52 μg/kg), the fact that bean is among the most important food items consumed in Brazil indicate the need for adoption of MPL for DON in this product.

ZEN was detected in only 2 samples (14.3%) of rice, at a mean concentration of 5.84 ± 1.33 μg/kg (Table 8). There is little information on the incidence of ZEA in rice in Brazil, although a recent study aiming to evaluate the presence of ZEN, AFB_1 and DON in rice from southern regions in Brazil indicated that only ZEN was significantly detected in 60% of samples at levels of 90.56 to 126.31 μg/kg (SAVI et al., 2018). Moreover, 36% of the samples analyzed in the study by Savi et al. (2018) were higher than the tolerance limit established by Brazilian regulations. However, our results are similar to those reported in white rice from South Korea (OK et al., 2014), in which ZEN was detected in 8.8% of samples at levels of 4.0-11.5 μg/kg.

3.4 Conclusion

The performance parameters of the analytical method were satisfactory, hence enabling its application for the simultaneous determination of all mycotoxins regulated in Brazil in corn meal, corn flour, wheat flour, rice and bean. By using the method in 36 samples of food products, high incidences of fumonisins in corn products along with DON in wheat flour were observed, although at concentrations below the Brazilian tolerance limits. Results indicated a low dietary exposure to mycotoxins in the small-scale farms evaluated, although the co-occurrence of fumonisins and DON warrants concern about their incidence in wheat flour in Brazil.

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4 Chapter III: **Co-occurrence of mycotoxins in maize food and maize-based feed from small-scale farms in Brazil: a pilot study**

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Abstract

A limited survey was conducted to assess the co-occurrence of aflatoxins (AF) B_1, B_2, G_1 and G_2, fumonisins (FB) B_1 and B_2, ochratoxin A (OTA), zearalenone (ZEN) and deoxynivalenol (DON) in maize food ($N = 26$) and animal feed ($N = 45$) collected from 21 small-scale farms from the states of São Paulo (SP) and Santa Catarina (SC), Brazil. Samples evaluated were maize meal and maize flour for human consumption available in the farm households, and maize-based feed intended for broiler chicks, laying hens and dairy cows. Analyses of mycotoxins were performed by ultra-performance liquid chromatography coupled with tandem mass spectrometry. The median levels of mycotoxins found in maize food were 2.5 μg/kg (total AF), 120 μg/kg (total FB), 13 μg/kg (ZEN), and 57 μg/kg (DON). All values were below the Brazilian tolerance limits, except for total FB in one sample of maize flour. In feed samples, median levels of total AF, total FB, ZEN and DON were 100 μg/kg, 680 μg/kg, 160 μg/kg and 200 μg/kg, respectively. The co-occurrence of two or more mycotoxins was confirmed in 35% and 51% of maize food and feed, respectively. Results indicate a low human exposure to mycotoxins in the small-scale farms evaluated, and a higher exposure of farm animals to mycotoxins in the feed.

Keywords: Mycotoxins; aflatoxins; ochratoxin A; fusariotoxins; occurrence; maize; feed.

4.1 Introduction

Mycotoxins are low molecular weight compounds with different chemical structures that are produced as secondary metabolites by several fungi species during growth on agricultural commodities, especially in cereal products (ABBAS, 2005). In Brazil, the most common toxigenic fungi found in cereals include species of the genera *Aspergillus* and *Fusarium*. *Aspergillus* species, mainly *A. flavus*, *A. parasiticus* and *A. nomius*, produce aflatoxins (AF) B_1, B_2, G_1 and G_2, which have high toxicity, teratogenicity, mutagenicity and carcinogenicity (OLIVEIRA et al., 2014). *A. ochraceus* (formerly *A. alutaceus*) produces ochratoxin A (OTA), a toxin that interferes with the synthesis of macromolecules in the cells of the renal parenchyma (PFOHL-LESZKOWICZ; MANDERVILLE, 2007). The mycotoxins produced by several species of the *Fusarium* genus, or fusariotoxins, include the fumonisins (FB) B_1 and B_2, deoxynivalenol (DON) and zearalenone (ZEN), among others, which exhibit a great variety of toxic effects in vertebrates including humans (BRYDEN, 2012). Two or more mycotoxins may be present in cereal products because of

multiple fungi contamination, or when one species produces more than one type of mycotoxin (DIAZ, 2005).

Maize and maize-based products are among the most vulnerable cereals to toxigenic fungi proliferation and subsequent contamination with single or multiple mycotoxins (BRYDEN, 2012). In Brazil, previous studies indicated that the most frequently occurring mycotoxins in commercially available maize food are the aflatoxins (JAGER et al., 2013; SOUZA et al., 2013) and fumonisins (BORDIN et al., 2015; MARTINS et al., 2012a). In particular, high frequencies (up to 100%) and levels (20-22,600 μg/kg) of FB_1 contamination have been reported in maize food from the Southern areas in Brazil in the past decade, including São Paulo (BORDIN et al., 2015), Paraná (MARTINS et al., 2012a; MORENO et al., 2009) and Santa Catarina states (SCAFF; SCUSSEL, 2004; WESTHUIZEN et al., 2003). Moreover, maize is the main ingredient (~60%) used in feed for broiler chicks, laying hens and dairy cows in Brazil (MARTINS et al., 2012b; OLIVEIRA et al., 2006). Consequently, the occurrence of mycotoxins in animal feed is usually associated with their frequencies and levels found in the maize used as an ingredient. AFB_1 and FB_1 are also the most frequently reported mycotoxins in commercial feed for poultry (OLIVEIRA et al., 2006) in Brazil, followed by OTA (BORDINI et al., 2016; MARTINS et al., 2012b) and ZEN (OLIVEIRA et al., 2006).

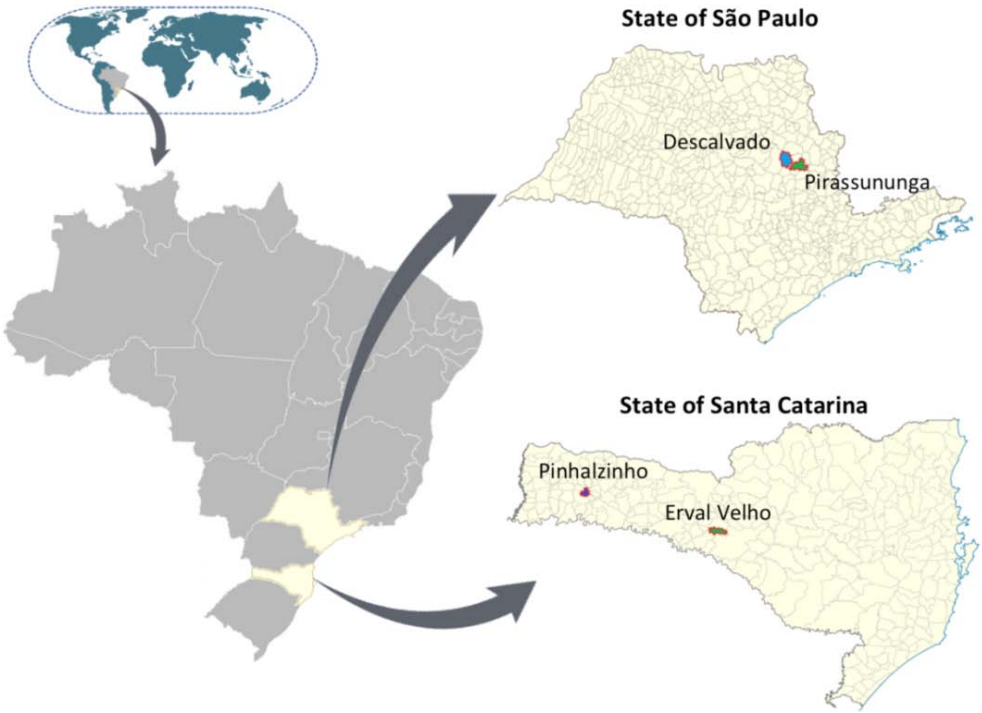
Brazilian regulations for mycotoxins in foods were revised in 2011, to include maximum permitted levels (MPL) for OTA, FB, ZEN and DON in several food products along with already established MPL for AF. For maize food, MPL established for total AF ($B_1 + B_2 + G_1 + G_2$), OTA, FB ($B_1 + B_2$), ZEN and DON are 20, 10, 1500, 150 and 750 μg/kg, respectively (ANVISA, 2011). However, there is no regulation for mycotoxins in feed in Brazil, except for a recommended maximum value of 50 μg/kg of total AF in feed ingredients (BRASIL, 1988). Additionally, there is very little information on multiple mycotoxin occurrences in feed prepared or available for animal consumption on farms, or in food products found in farm households in Brazil. The aim of the present study was to conduct a limited survey on the co-occurrence of regulated mycotoxins in maize food for human consumption and in maize-based feed intended for poultry and dairy cows in small-scale farms from Southern states in Brazil.

4.2 Material and methods

4.2.1 Sampling design

The study was conducted from April to December 2016 on 21 small-scale dairy and poultry farms: 8 farms located in the surroundings of Descalvado and Pirassununga cities, Northeast region of the state of São Paulo; and 13 farms in the nearby of municipalities of Pinhalzinho and Erval Velho, Western region of the state of Santa Catarina (Figure 4). The climates in both regions are classified as humid subtropical, with slight differences in their annual mean temperatures and rainfalls: 18-20º C and 1300-1600 mm without dry season in São Paulo, respectively; 16-18º C and 1600-1900 mm with dry winter in Santa Catarina, respectively (ALVARES et al., 2013). These regions were selected because of their large number of small-scale, family-operated farms in their respective states. In each region, the distances between farms varied from 20 to 100 km. All farms had primarily poultry facilities for raising broilers and/or laying hens, although some also had dairy cows as a secondary animal production activity.

Figure 4 - Location of the 4 cities in Brazil for collection of corn products and feed



Source: Own authorship.

In each farm, two sampling procedures were conducted, one for maize food available in the households and another for maize-based feed disposed in feed troughs. For maize

food, approximately 200 g of maize meal and maize flour were collected. The majority of maize food collected were industrialized products that were previously purchased by farmers (not manufactured in the farm) in supermarkets in nearby cities and were available in their original packages (0.5-1.0 kg) in the households at the time of sampling. Composite samples of feed were collected from 3-5 places in the feed troughs until nearly 400 g. All the feed evaluated in the experiment were maize-based, in-farm manufactured (not purchased by the farmers) and had no visible signs of fungal infection. A total of 71 samples were collected at the end of the sampling period: 26 samples of maize food and 45 samples of maize-based feed. Because not all types of maize food or feed were available in each farm visited, the number of samples of each type of food or feed varied according to their availability in the 21 farms visited. Samples of maize meal (N = 11), maize flour (N = 15), feed for dairy cows (N = 15), grower feed for broiler chicks (N = 10) and feed for mature laying hens (N = 20) were placed in polyethylene bags and taken immediately to the laboratory. Upon arrival, all food and feed samples were homogenized, finely milled and frozen at -20 °C until analysis for mycotoxins.

4.2.2 Reagents and solutions

All reagents were of analytical grade and water was purified by deionization (Milli-Q system, Millipore, Bedford, MA, USA). HPLC-grade acetonitrile and methanol (J.T. Baker, Phillipsburg, NJ) were used for chromatographic analyses. Mycotoxin standards (AFB_1, AFB_2, AFG_1, AFG_2, OTA, FB_1, FB_2, ZEN and DON) were purchased from Sigma (Sigma, St Louis, MO, USA). Individual stock solutions of unlabeled standards (US) of each mycotoxin were prepared at 10 µg/mL in water/acetonitrile (1:1, v/v). After that, convenient aliquots of each mycotoxin stock solution were transferred to a vial in order to obtain a 1-mL US working solution containing a mixture of DON (750 ng/mL), AFB_1, AFB_2, AFG_1, AFG_2, OTA, FB_1, FB_2 and ZEN (100 ng/mL) in water/acetonitrile (1:1, v/v). Isotopically labeled standards (IS) of [$^{13}C_{17}$]-AFB_1 (Sigma, St. Louis, MO), [$^{13}C_{20}$]-OTA, [$^{13}C_{34}$]-FB_1, [$^{13}C_{18}$]- ZEN and [$^{13}C_{15}$]-DON (Biopure, Romer Labs, Tulln, Austria) were also used in the analyses. Each IS was individually diluted to 1.0 µg/mL in water/acetonitrile (1:1, v/v) and used to prepare a IS working solution containing [$^{13}C_{17}$]-AFB_1, [$^{13}C_{20}$]-OTA and [$^{13}C_{34}$]-FB_1 at 5 ng/mL, [$^{13}C_{18}$]- ZEN at 250 ng/mL, and [$^{13}C_{15}$]-DON at 500 ng/mL in water/acetonitrile (1:1, v/v). Both US and IS working solutions were stored at -20^{o}C until analysis.

4.2.3 Sample preparation

The procedures for extraction of mycotoxins from maize food and maize-based feed were conducted according to the methodology as described by Sulyok, Krska e Schuhmacher (2007), with some modifications. Briefly, the previously homogenized samples were weighed (1.0 g) in 15-mL Falcon tubes in duplicate, and 4 mL of the extraction solvent containing acetonitrile/water/acetic acid (80:20:1%, v/v/v) were added to the tubes. The tubes were vortexed for 1 min and placed in an orbital shaker (Tecnal, Piracicaba, Brazil) for 60 min. The samples were re-vortexed, and shaken for an additional 30 min. The samples were then centrifuged at 1,500 x g for 5 min, and the supernatants were filtered through 0.22 μm PTFE membranes (Millipore, Burlington, MA). An 80-μL aliquot of the supernatant was placed in a glass insert (properly inserted into a vial), thoroughly mixed with 20 μL of the IS working solution prepared as previously described and reserved for further UPLC-MS/MS analysis. Each vial with the final sample extract contained 1.0 ng/mL of [$^{13}C_{17}$]-AFB_1, [$^{13}C_{20}$]-OTA and [$^{13}C_{34}$]-FB_1, 50 ng/mL of [$^{13}C_{18}$]- ZEN and 100 ng/mL of [$^{13}C_{15}$]-DON.

4.2.4 Liquid chromatographic and mass spectrometry

Final extracts containing IS working solution were injected into a Waters Acquity I-Class ultra-performance liquid chromatographic (UPLC) system (Waters, Milford, MA, USA) equipped with a BEH C_{18} column (2.1 × 50 mm, 1.7 μm) and coupled to a Xevo® TQ-S mass spectrometer (MS/MS) (Waters, Milford, MA, USA). For chromatographic separation, 3 μL of extracted samples and standards were injected into the UPLC-MS/MS system. The column was kept at 40°C during the analyses, and samples were maintained at 15°C. The mobile phase was composed of water (eluent A) and acetonitrile (eluent B), both containing 0.1% of acetic acid and ammonium acetate at 5 mM. The percentage of eluent A was kept at 98% for 1 min. and the percentage of eluent B was linearly increased to 30% over 5 min. and then increased to 96% over 2 min, followed by a hold time of 0.5 min. After that, the percentage of eluent B was reduced to 2% over 0.5 min and the column re-equilibrated to the initial conditions for 2 min. Total chromatographic run time was 10 min, and the mobile phase flow rate was maintained at 0.6 mL/min.

The mass spectrometer was operated in the Multiple Reaction Monitoring (MRM) mode using electrospray ionization in either positive or negative ion mode. Mass spectrometric parameters were as follows: capillary voltage: 3.00 kV for the positive mode, and 2.00 kV for the negative mode; source temperature: 150°C; desolvation temperature:

500°C, desolvation gas flow: 700 L/h; nebulizer gas: 7.00 bar and cone gas flow 150 L/h. Cone voltage, collision energy, and MRM transitions (major precursor ion > fragment ion) were manually optimized for individual mycotoxins. Quantification and confirmatory MRM transitions for mycotoxins and corresponding optimal mass spectrometric parameters used in the LC-MS/MS analysis are presented in Table 9. Data collection and processing was performed using software MassLynx version 4.1.

Table 9 - Mass spectrometry conditions used for mycotoxins analyses in maize food and maize-based feed samples by Multiple Reaction Monitoring

Mycotoxin	Mass (g/mol)	Molecular ion	Transition (*m/z*)	Cone voltage (V)	Collision energy (V)
AFB_1	312.28	$[M+H]^+$	312.7 > 284.9 [a]	94	36
			312.7 > 241.1 [b]		22
$[^{13}C_{17}]$-AFB_1	329.15	$[M+H]^+$	330.3 > 301.5	94	20
AFB_2	314.29	$[M+H]^+$	314.7 > 259.0 [a]	2	28
			314.7 > 287.0 [b]		26
AFG_1	328.27	$[M+H]^+$	328.9 > 243.0 [a]	2	26
			328.9 > 199.5 [b]		38
AFG_2	330.29	$[M+H]^+$	330.9 > 245.0 [a]	56	28
			330.9 > 188.9 [b]		40
OTA	403.08	$[M+H]^+$	404.0 > 238.9 [a]	35	22
			404.0 > 357.9 [b]		12
$[^{13}C_{20}]$-OTA	423.67	$[M+H]^+$	424.2 > 250.0	30	20
FB_1	721.84	$[M+H]^+$	722.5 > 334.0 [a]	50	40
			722.5 > 352.1 [b]		30
$[^{13}C_{34}]$-FB_1	755.58	$[M+H]^+$	756.6 > 374.4	50	30
FB_2	70.83	$[M+H]^+$	706.5 > 336.2 [a]	50	35
			706.5 > 318.3 [b]		40
ZEN	318.15	$[M-H]^-$	317.1 > 175.1 [a]	50	23
			317.1 > 130.9 [b]		33
$[^{13}C_{18}]$-ZEN	336.23	$[M-H]^-$	335.1 > 185.1	50	31
DON	296.32	$[M+H]^+$	397.3 > 249.1 [a]	6	10
			397.3 > 231.1 [b]		14
$[^{13}C_{15}]$-DON	311.21	$[M+H]^+$	312.1 > 98.7	30	35

Source: Own authorship. [a] Transitions used for quantification. [b] Transitions used for confirmation.AF: aflatoxin; OTA: ochratoxin A; FB: fumonisin; ZEN: zearalenone; DON: deoxynivalenol.

Calibration curves were generated with mycotoxin standards prepared in triplicate using serial dilutions of the US working solution prepared as previously described, at the following levels in water/acetonitrile (9:1, v/v): 0.05, 0.25, 0.5, 1.0, 2.0, 3.0 and 4.0 ng/mL for AFB_1, AFB_2, AFG_1, AFG_2, OTA, FB_1, FB_2 and ZEN; 0.37, 1.9, 3.7, 7.5, 15, 23 and 30 ng/mL for DON. Additionally, 20 µL of the IS working solution was added to 80 µL of each calibration point. Each calibration curve was constructed by plotting the relative response (the ratio of the peak area of each US standard to the peak area of the corresponding IS) as a function of the nominal concentration of the US standard. The concentration of each analyte in samples was calculated by the relative response and the calibration curves using internal calibration. Thus, suppression or increase of the analyte signal caused by possible matrix effects of the sample extract was compensated by the corresponding signal variation of the IS signal. The limits of quantification (LOQ) and detection (LOD) were calculated based on signal-to-noise ratios of 3:1 and 10:1, respectively, of peaks corresponding to the confirmatory MRM transitions (EUROPEAN COMMISSION, 2006a).

4.2.5 Evaluation of analytical method´s performance

The performance characteristics of the analytical method were evaluated by using blank samples of each type of maize food and maize-based feed. Parameters evaluated included the limits of detection (LOD) and quantification (LOQ), apparent recovery (R_A), linearity, signal suppression/enhancement (SSE) due to matrix effects and extraction recovery (R_E). LOQ and LOD were calculated based on signal-to-noise ratios of 3:1 and 10:1, respectively, of peaks corresponding to the confirmatory MRM transitions. R_A, SSE and R_E were determined based on calibration curves constructed from the analytical data obtained in samples spiked before extraction, spiked extracts and standards diluted in solvent, considering the addition of the IS working solution in all prepared samples.

Spiked samples were prepared by adding appropriate volumes of US working solution to triplicate tubes containing blank samples (1.0 g) before extraction, to achieve the following levels of mycotoxins: 1.2, 2.5, 5.0, 10, 15 and 20 µg/kg for AFB_1, AFB_2, AFG_1, AFG_2, OTA, FB_1, FB_2 and ZEN; 9.3, 19, 37, 75, 110 and 150 µg/kg for DON. The tubes were kept uncapped overnight in the dark at room temperature to allow solvent evaporation. On the following two days, the tubes were capped and shaken by hand twice a day to ensure proper homogenization between the analytes and matrix. Next, spiked samples were submitted to the same extraction procedures as described for samples collected in the farms. To prepare spiked extracts, blank samples (1.0 g) of each type of product were extracted

and matrix-matched calibration curves were prepared by spiking the resulting blank extracts with appropriate volumes of the US working solution to reach concentrations of 0.25, 0.5, 1.0, 2.0, 3.0 and 4.0 ng/mL for AFB_1, AFB_2, AFG_1, AFG_2, OTA, FB_1, FB_2 and ZEN; 1.9, 3.7, 7.5, 15, 23 and 30 ng/ mL for DON. Triplicate spiked extracts were prepared for each mycotoxin level. Triplicate US diluted in solvent were prepared in water/acetonitrile (9:1, v/v) at the same levels of spiked extracts. Eighty L of each level of final extracts from spiked samples, spiked extracts and liquid standards were combined with 20 L of the IS working solution in glass inserts inside amber vials, and stored at -20ºC until analysis by LC-MS/MS. R_A, SSE and R_E values were calculated according to equations (Eq. 1), (Eq. 2) and (Eq. 3), respectively, as proposed by Sulyok et al. (2006) and Varga et al. (2012).

$$R_A\ (\%) = 100^* \text{ slope}_{\text{ spiked sample}} / \text{slope}_{\text{ standard diluted in solvent}} \quad \text{(Eq. 1)}$$

$$\text{SSE } (\%) = 100^* \text{ slope}_{\text{ spiked extract}} / \text{slope}_{\text{ standard diluted in solvent}} \quad \text{(Eq. 2)}$$

$$R_E\ (\%) = 100^* R_A / \text{SSE} \quad \text{(Eq. 3)}$$

4.3 Results and discussion

4.3.1 Performance of the analytical method

The retention times, LOD and LOQ, R_A, SSE and R_E values for each mycotoxin in samples of maize food and maize-based feed for poultry and dairy cows are shown in Table 10. Overall, the calculated LOQ values for the individual mycotoxins varied between 0.6 to 18 μg/kg, which were much lower than their respective MPL values in foods established by the Brazilian regulations (ANVISA, 2011). R_A and SSE values for the mycotoxins varied from 92 to 109% and from 89 to 107%, respectively. The determined R_E were high for all mycotoxins (96–110%), which fulfil the requirements described by the European Commission (2006a).

R_A, SSE and R_E parameters were determined in all types of samples considering the addition of IS in the samples, aiming to verify its ability to compensate the matrix effects (VARGA et al., 2012). The R_A percentage represents the ratio of an observed value obtained from a calibration curve (prepared in spiked samples) and a reference known or theoretical value (in this case, the calibration curve prepared with US in solvent). SSE is calculated by comparison of the respective matrix-matched US standards with the US in solvent, and R_E expresses the yield of the extraction step (SULYOK et al, 2006). The performance parameters obtained in the present study indicate that the addition of IS efficiently

compensated the signal suppression or enhancement, hence confirming the suitability of the analytical method for quantification of the evaluated mycotoxins in maize food and maize-based feed.

Table 10 - Method performance parameters for determination of mycotoxins in samples of maize food and maize-based feed [1]

Mycotoxin	RT (min)	Concentration range (μg/kg)	R_A range (%)	SSE range (%)	R_E range (%)	LOD (μg/kg)	LOQ (μg/kg)
AFB_1	4.80	1.2-20	101-102	92-93	110	0.4	0.9
AFB_2	4.50	1.2-20	100	93-94	106-107	0.4	0.8
AFG_1	4.46	1.2-20	105-109	103-107	101-102	0.4	0.8
AFG_2	4.18	1.2-20	99	95	104	0.5	1.0
OTA	5.99	1.2-20	97	92-94	103-105	0.5	1.0
FB_1	5.40	1.2-20	105	95-101	103-110	0.9	2.5
FB_2	3.74	1.2-20	92	96	96	0.7	2.0
ZEN	5.98	1.2-20	103	105	98	0.2	0.6
DON	1.98	9.3-150	93	89-91	102-104	6.1	18

Source: Own authorship. [1] $n = 3$ per type of sample, in each spiked level.
RT: Retention time; R_A: Apparent recovery; SSE: Signal suppression/enhancement; R_E: Extraction recovery; LOD: Limit of detection; LOQ: Limit of quantification. AF: aflatoxin; OTA: ochratoxin A; FB: fumonisin; ZEN: zearalenone; DON: deoxynivalenol.

4.3.2 Occurrence of mycotoxins in maize food and maize-based feeds

Table 11 presents the mycotoxin levels found in samples of maize food (maize flour and maize meal) collected on small-scale farms from São Paulo and Santa Catarina states, along with the MPL adopted in Brazil for each mycotoxin. Twenty-four samples (92%, $N = 26$) were positive for at least one of the mycotoxins analyzed, with higher frequencies of *Fusarium* mycotoxins (FB, DON and ZEN). AFB_1 or AFG_1 were detected in only 1 sample (4%), and AFB_2, AFG_2, and OTA were not detected in any food sample analyzed. FB_1 was the most frequent mycotoxin in maize food, being detected in 24 samples (92%) at levels ranging from 2.9 to 880 μg/kg (median: 110 μg/kg). Several studies have also demonstrated high FB occurrence in Brazilian maize food (BORDIN et al., 2015; SCAFF; SCUSSEL, 2004; WESTHUIZEN et al., 2003). FB_1 was found in 73% of the maize food analyzed, including maize flour, popcorn, polenta, canned maize and cornflakes (BORDIN, et al., 2015). In our study, despite the high frequency of FB in maize food, the levels were much

lower than the MPL adopted in Brazil, with the exception of one sample of maize flour that exceeded the tolerance limit of 1500 μg/kg for the sum of FB_1 and FB_2 (ANVISA, 2011).

Table 11 - Mycotoxin levels in samples of maize food (maize meal and maize flour, N = 26) collected in small-scale farms from the states of São Paulo and Santa Catarina, Brazil

Mycotoxin	*n*	%	Concentration range (μg/kg)	Median (μg/kg)	MPL (μg/kg)	*n* > MPL
AFB_1	1	4	-	3.6	NE	-
AFB_2	0	0	-	-	NE	-
AFG_1	1	4	-	1.3	NE	-
AFG_2	0	0	-	-	NE	-
Σ AF	2	8	1.3 - 3.6	2.5	20	0
OTA	0	0	-	-	10	0
FB_1	24	92	2.9 - 880	110	NE	-
FB_2	10	38	5.9 - 630	88	NE	-
Σ FB	25	96	2.9 - 1500	120	1500	1
ZEN	4	15	8.7 - 180	13	150	1
DON	8	31	44 - 79	57	750	0

Source: Own authorship. *n*: Number of samples showing concentrations above the limit of quantification (LOQ) - See Table 10 for LOQ values. MPL: Maximum permitted levels adopted by Brazilian regulations (Agência Nacional de Vigilância Sanitária, 2011). AF: aflatoxin; OTA: ochratoxin A; FB: fumonisin; ZEN: zearalenone; DON: deoxynivalenol. NE: Not established.

DON was detected in 31% of the samples but at low levels ranging from 44 to 79 μg/kg (median: 57 μg/kg), while ZEN was found in 15% of the samples with only one sample of maize flour exceeding the MPL of 150 μg/kg (ANVISA, 2011). These results are different from data reported in a previous study showing that ZEN was detected in only one sample of maize-based food traded in municipalities of the State of Paraná (ALMEIDA-FERREIRA et al., 2013). Rodrigues and Naehrer (2012) reported frequencies of 17% (*N* = 322) and 43% (*N* = 321) for DON and ZEN, respectively, in maize samples surveyed in South America (93% of the samples were collected in Brazil). Taking into account that the majority of maize food collected in the present study were industrialized products that were previously purchased by farmers (not manufactured in the farm), the results indicate an overall compliance of maize meal and flour to the MPL adopted in Brazil.

The levels of mycotoxins in maize-based feed (broiler chicks, laying hens and dairy cows) are presented in Table 12, including the MPL or guidance levels established for mycotoxins in Brazil and in the European Union.

Table 12 - Mycotoxin levels in maize-based feed samples for broiler chicks, laying hens and dairy cows (N = 45) collected in small-scale farms from the states of São Paulo and Santa Catarina, Brazil

Mycotoxin	*n*	%	Concentration range (μg/kg)	Median (μg/kg)	MPL/GL (μg/kg)	*n* > MPL/GL
AFB_1	6	13	8.7 - 390	100	20 [1] 5 [2]	4 2
AFB_2	2	4	4.2 - 5.4	4.8	NE	-
AFG_1	2	4	2.6 - 12	7.1	NE	-
AFG_2	0	0	-	-	NE	-
Σ AF	6	13	8.7 - 400	100	50 [3]	4
OTA	1	2	-	11	100 [4]	0
FB_1	42	93	17 - 53,000	350	NE	-
FB_2	39	87	33 - 2,800	360	NE	-
Σ FB	42	93	17 - 54,000	680	20000 [4] 50000 [5]	1 0
ZEN	13	29	27 – 520	160	500 [5]	1
DON	20	44	46 - 2,300	200	5000 [6]	0

Source: Own authorship. [1,2] Maximum permitted levels for complete feedingstuffs for poultry and dairy animals, respectively (EUROPEAN COMMUNITIES, 2002). [3] Maximum level recommended by Brazilian regulations (BRASIL, 1988). [4] Guidance levels for complete feedingstuffs for poultry (EUROPEAN COMMISSION, 2006b). [5] Guidance levels for feed materials for adult ruminants (EUROPEAN COMMISSION, 2006b). [6]Guidance levels for any complete feedingstuffs (EUROPEAN COMMISSION, 2006b).
n: Number of samples showing concentrations above the limit of quantification (LOQ) - See Table 10 for LOQ values. MPL/GL: Maximum permitted levels (MPL) or guidance levels (GL). AF: aflatoxin; OTA: ochratoxin A; FB: fumonisin; ZEN: zearalenone; DON: deoxynivalenol. NE: Not established.

At least one type of mycotoxin was found in 44 samples analyzed (98%, $N = 45$). The AF were detected in 13% of total feeds analyzed ($N = 45$), and four feed samples had total AF levels higher than the Brazilian recommended level (50 μg/kg) for any type of feed (BRASIL, 1988), with a median of 100 μg/kg. Considering only AFB_1, four samples of feed for poultry and two of feed for dairy animals had much higher levels than the MPL established by the Directive 2002/32/EC for those types of feed (European Communities

2002). As observed for maize food (Table 11), FB (B_1 and B_2) had higher frequencies (93%) in feed, with medians of 350 and 360 μg/kg, respectively (median of total FB: 680 μg/kg). One sample of feed for poultry containing 54,000 μg/kg would not comply with the European standard for total FB (20,000 μg/kg) in this type of feed (EUROPEAN COMMISSION, 2006a). ZEN and DON were found in 29% and 44% of samples analyzed, with median values of 160 μg/kg and 200 μg/kg, respectively. All levels of DON found in positive samples of feed were below the European recommendations (5,000 μg/kg), and one sample of feed for dairy cow had ZEN concentration (520 μg/kg) higher than the European guidance level (500 μg/kg) (EUROPEAN COMMISSION, 2006b).

Previous studies have demonstrated the occurrence of mycotoxins (AFs, ZEN, DON and FB) in animal feed from Brazil. Similarly, to our study, high frequencies of FB at levels up to 43,800 μg/kg have been reported (MACHINSKI; SOARES, 2000; POZZI et al., 1995; SALAY; MERCADANTE, 2002). In addition to concerns over adverse effects from direct consumption of mycotoxin-contaminated food, there is also a risk of ingestion of animal-derived food products, such as meat, milk, or eggs which may contain residues or metabolites of mycotoxins (ALSHANNAQ; YU, 2017). In particular, the excretion of aflatoxin M_1 in milk is a very important human health concern especially for children that are the largest consumers of milk, and more susceptible to toxic compounds (FLORES-FLORES et al., 2015). Consequences of the high levels of some mycotoxins observed in feed are difficult to assess at this time, since other variables (e.g., disease incidences, productivity parameters) were not measured in the experiment. Maize produced or stored in the farms was probably the main source of mycotoxin contamination of feed, considering that it is the main ingredient of feed for poultry and dairy cows (~60% of the total feed composition), and that all the feed evaluated were in-farm manufactured. Thus, the high levels of AFB_1 and FB_1 observed in this study emphasize the importance of improving the control of mycotoxin levels, as well as the need for adoption of regulations for mycotoxins in feed in Brazil.

Table 13 presents the co-occurrence of mycotoxins in maize food and maize-based feeds, considering all samples showing concentrations above the LOQ for each type of mycotoxin. Overall, 32 samples (45%, $N = 71$) had 2 – 5 quantifiable mycotoxins, which comprised of 9 samples of maize food (35%, $N = 26$) and 23 samples of maize-based feed (51%, $N = 45$). FB and DON were the main mycotoxins mixture found in samples of maize

food (n = 8) or feed (n = 18), although they were frequently associated with ZEN, especially in feed samples.

Table 13 - Mycotoxin co-occurrence in samples of maize food (maize meal and maize flour) and maize-based feed for broiler chicks, laying hens and dairy cows collected in small-scale farms from the states of São Paulo and Santa Catarina, Brazil

Number of mycotoxins	Types of co-occurring mycotoxins	Number of positive samples [1]	
		Maize food (N = 26)	Maize-based feed (N = 45)
2	AF, FB	1	3
	FB, ZEN	0	2
	FB, DON	5	0
	FB, DON	0	6
3	AF, FB, DON	0	1
	FB, ZEN, DON	2	2
	FB, ZEN, DON	0	7
4	AF, FB, ZEN, DON	1	1
5	AF, FB, OTA, ZEN, DON	0	1
	Total (%)	9 (35)	23 (51)

Source: Own authorship. [1] Samples showing concentrations above the limit of quantification (LOQ) - See Table 10 for LOQ values. AF: aflatoxin; OTA: ochratoxin A; FB: fumonisin; ZEN: zearalenone; DON: deoxynivalenol.

Despite the limited information on the co-occurrence of fusariotoxins in Brazil, these results are in agreement with data reported by Souza et al. (2013), who observed that 4% and 12% of samples of maize and poultry feed samples were positive for DON and ZEN, respectively. The combination of multiple mycotoxins may cause additional adverse effects relative to a single mycotoxin exposure, with additive or synergistic interactions (ALASSANE-KPEMBI et al., 2017). However, mycotoxins regulations have not addressed the co-occurrence of mycotoxins in food or feed products (EFSA, 2013).

In conclusion, results of this limited survey indicated high frequencies and low levels of mycotoxins in the maize food evaluated, except for two samples containing FB and ZEN at concentrations above their respective Brazilian MPL. Much higher frequencies and/or levels were observed in maize-based feed, hence emphasizing the need to improve the feed quality regarding the contamination with mycotoxins in small-scale farms in Brazil, especially for AF, FB and ZEN. Furthermore, the co-occurrence of 2 or more mycotoxins

in 45% of samples analyzed warrants concern about their frequencies in maize food and animal feed in Brazil.

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5 Chapter IV: **Assessment of Mycotoxin Exposure and Risk Characterization Using Occurrence Data in Foods and Urinary Biomarkers in Brazil**

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Abstract

This study aimed to assess the exposure of residents (*N* = 86) from rural areas of two Brazilian states, São Paulo and Santa Catarina, to multiple mycotoxins and to characterize the associated risk. The volunteers provided urine (*N* = 162) and cereal-based food samples (*N* = 203) available from their homes and in two sampling periods (April-May and December/2016). The incidences of mycotoxins exceeding the Brazilian maximum permitted levels (MPL) were 1.5% (*N* = 66) for zearalenone (ZEN) in rice, 13% (*N* = 39) for deoxynivalenol (DON) in wheat flour, 5% (*N* = 21) for total fumonisins (FB) and 11% (*N* = 18) for ZEN in corn flour. Aflatoxins (AF) M_1 and AFP_1, DON, ochratoxin A (OTA), FB_1, and ZEN were detected in urine samples at levels of 0.02-12.0 ng/mg creatinine. The mean probable daily intake (PDI) values based on occurrence data in foods varied from 0.007 to 0.013, 0.069 to 1.002, 0.119 to 0.321 and 0.013 to 0.156 μg/kg body weight (b.w.)/day for AFs, DON, FBs and ZEN, respectively. Mean PDI values based on urinary biomarkers were 0.001, 84.914, 0.031, 0.377 and 0.002 μg/kg b.w./day for AFB_1, DON, OTA, FB_1 and ZEN, respectively. Hazard quotient (HQ) based on occurrence data in food indicated a potential health concern (> 1) for ZEN in the 2nd sampling. HQ values > 1 based on urinary biomarkers were observed for DON in the two sampling periods. Although OTA was not detected in any food sample, the HQ value based on urinary OTA levels was > 1 in the 1st sampling period. Margin of exposure values below 10,000 for AF resulted from food and urine data in the 1st sampling, indicating potential health risks. Future studies are needed to assess additional food as well as non-food sources of mycotoxin exposure in the populations studied.

Keywords: Mycotoxins. Biomarkers. Urine. Food. Risk assessment. LC-MS/MS.

5.1 Introduction

Mycotoxins are organic compounds of low molecular weight produced as secondary metabolites by various species of fungi during growth on foodstuffs. They present different chemical structures and occur in several agricultural products, especially cereals (ABBAS, 2005). The most well-know and studied mycotoxins are the aflatoxins (AF), deoxynivalenol (DON), ochratoxin A (OTA), fumonisins (FB), and zearalenone (ZEN). *Aspergillus* species (mainly *A. flavus*, *A. parasiticus* and *A. nomius*) are the main producers of the most important, highest toxic AF compounds (AFB_1, AFB_2, AFG_1 and AFG_2) showing teratogenic, mutagenic and carcinogenic effects in several animal species and humans

(JAGER et al., 2013). DON, also called vomitoxin, is a type B trichothecene mainly produced by *Fusarium roseum*, which causes nausea, diarrhea, reduced nutritional efficiency, gastrointestinal tract injuries, and weight loss in animals (OLIVEIRA et al., 2014). OTA, which is produced by *Aspergillus* and *Penicillium* (ABBAS, 2005), interferes with the synthesis of macromolecules in the cells of the renal parenchyma, including DNA, RNA, and proteins. *F. verticillioides* produces FB_1, as the most toxic compound, FB_2 and FB_3 predominantly found in natural conditions (OLIVEIRA et al., 2014). ZEN is an estrogenic substance derived from resorcylic acid produced by several *Fusarium* species, such as *F. roseum (F. graminearum), F. culmorum,* and *F. equisetum* (MAGAN; OLSEN, 2006).

Due to the risks posed to human health, several countries, including Brazil, have set maximum permitted levels (MPL) for mycotoxins in different food products. Brazilian regulations for mycotoxins determine MPL for total AF (sum of AFB_1, AFB_2, AFG_1 and AFG_2), DON, OTA, total FB (sum of FB_1 and FB_2), and ZEN in the most susceptible foods for contamination, including cereals and cereal-based products, such as rice, bean, wheat flour, corn flour, and corn meal (ANVISA, 2011). Despite regulations, the occurrence of mycotoxins in Brazilian food products has been highlighted by several studies, indicating high frequencies and concentrations of total AF, total FB and, more recently, DON, especially in corn, peanuts, wheat, and products made with these cereals (ALMEIDA-FERREIRA et al., 2013; DEL PONTE; GARDA-BUFFON; BADIALE-FURLONG, 2012; MACHINSKI et al., 2001; MARTINS et al., 2012a; MORENO et al., 2009; OLIVEIRA et al., 2009; PIACENTINI et al., 2015; SCAFF; SCUSSEL, 2004; SIMAS et al., 2007). Although the published data indicate a high potential for consumption of food products contaminated with more than one mycotoxin in Brazil, there is no published study on the exposure assessment or risk characterization, the two main steps of risk assessment, regarding the presence of multiple mycotoxins in the diet.

Exposure assessment can be determined by 2 different approaches, one indirect by combining food consumption and occurrence data, and another, direct approach based on urine biomarkers. In both approaches, exposure assessment is expressed as probable daily intake (PDI). For the risk characterization, the outputs of exposure, namely the daily intake values, are compared with the reference dose (ASSUNÇÃO et al., 2015). Exposure assessments based on food consumption and occurrence data have important limitations due to the heterogeneous distribution of mycotoxins in foods and to the limited accuracy of food consumption data (HEYNDRICKX et al., 2015). These caveats may be overcome with the

measurement of specific urinary biomarkers to assess exposure to mycotoxins, since biomarker excretion correlates well with the intake of some mycotoxins (GROOPMAN; KENSLER, 1999; QIAN et al., 1994). Urinary biomarkers suitable for AFB_1 and ZEN are AFM_1, AFP_1 and AFQ_1 (GROOPMAN; KENSLER, 1999), and non-metabolized ZEN + α-zearalenol (α-ZEL) + β-zearalenol (β-ZEL) (SOLFRIZZO; GAMBACORTA, 2014), respectively. Non-metabolized FB_1, OTA and DON + de-epoxideoxynivalenol 1 (DOM-1) + 15-acetyl-DON (15-Ac-DON) are urinary biomarkers for FB_1, OTA and DON, respectively (SOLFRIZZO; GAMBACORTA, 2014). Because these biomarkers are excreted in urine as free and conjugated forms, urine samples are generally digested with β-glucuronidase/sulfatase to deconjugate the conjugated forms, to increase the concentration and detectability of the free analytes (SOLFRIZZO et al., 2011). In this context, human biomonitoring of urinary biomarkers of mycotoxins may provide adequate exposure data for a more accurate risk characterization of mycotoxins in foods.

Human biomonitoring studies using urinary biomarkers for mycotoxins in Brazil have only been carried out for aflatoxins, and were based on the assessment of AFM_1 (JAGER et al., 2014; 2016; ROMERO et al., 2010) and AFB_1-N^7-guanine in urine (JAGER et al., 2016), which is a biomarker of early carcinogenic effects of AFB_1 (QIAN et al., 1994). However, previous studies have shown high occurrences of other regulated mycotoxins in food, indicating a potential high exposure in the Brazilian population, especially in rural areas (BORDIN et al., 2015; FRANCO et al., 2018; SCOZ et al., 2009; VAN DER WESTHUIZEN et al., 2003). Thus, the present study aimed to assess the exposure of volunteers of rural properties of São Paulo (SP) and Santa Catarina (SC), Brazil, to multiple mycotoxins by using consumption and food contamination data and mycotoxin urine biomarkers. Risk characterization was additionally performed to elucidate potential health concern.

5.2 Materials and methods

5.2.1 Subject background information

Participants were recruited among residents in small-scale dairy and poultry farms located in the surroundings of Descalvado and Pirassununga cities, Northeast region of the state of São Paulo, and of Pinhalzinho and Erval Velho, Western region of the state of Santa Catarina. The states of São Paulo and Santa Catarina were chosen because they have large number of family-operated farms with a centralized system of regulation and control. The climates in both regions are classified as humid subtropical, with slight differences in their

annual mean temperatures and rainfalls: 18-20º C and 1300-1600 mm without dry season in São Paulo, and 16-18º C and 1600-1900 mm with dry winter in Santa Catarina (ALVARES et al., 2013). The farms from the state of São Paulo contained silos for grain drying. Storage of cereals, in most cases, was carried out in sealed sheds protected from the rain. In the state of Santa Catarina, farms were simpler, with most grains stored in old sheds that showed cracks and had no protection against the entry of water, insects, and animals.

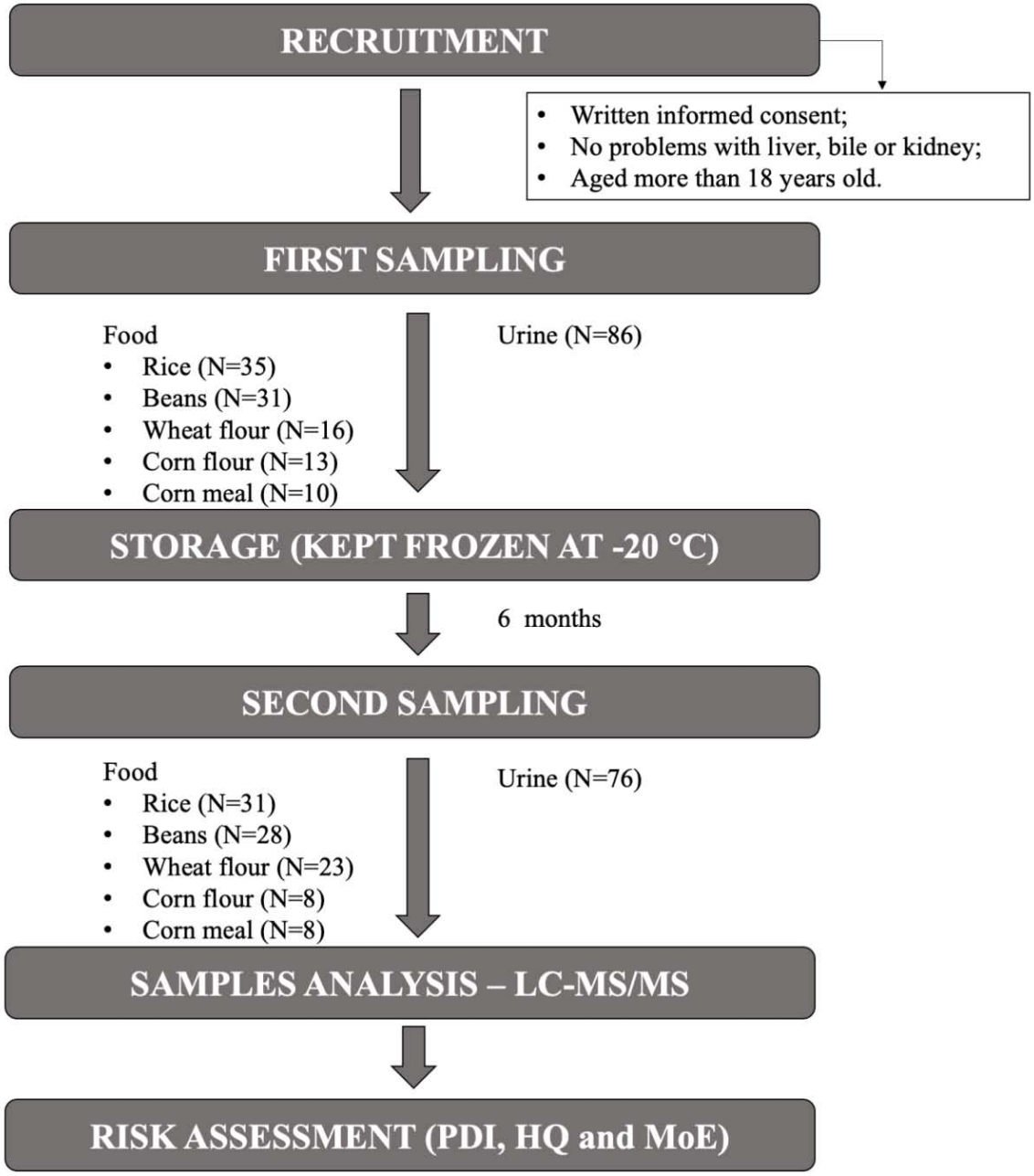
The study was submitted to and approved by the Research Ethics Committee (REC) of the School of Animal Sciences and Food Engineering, University of São Paulo (Opinion No. 1.500.317 – ANNEX A). All volunteers ($N = 86$) were older than 18 years of age (mean of 46.6 ± 17.0 years old),and included 44 women (51.2%) and 42 men (48.8%), with average weight 73.4 ± 15.8 kg. Before starting the experiment, they were invited to sign a Free and Informed Consent Form (ANNEX B) that was approved by the aforementioned REC, and to answer general questions about their health status. People with signs and/or symptoms of liver or kidney illness or any chronic disease were not included in the study, due to potential interferences with the metabolism of mycotoxins and creatinine.

5.2.2 Sampling design

Two sampling procedures were carried out, one during April and May (Brazilian Fall), and the other 7-8 months after that, in December (Summer). The total number of small-scale farms visited was 32, and the number of volunteers in the first sampling was 86 (30 in São Paulo and 53 in Santa Catarina). In the second sampling, 76 volunteers agreed to participate in the study (24 in São Paulo and 52 in Santa Catarina). Samples of rice ($N = 66$), bean ($N = 59$), wheat flour ($N = 39$), corn flour ($N = 21$) and corn meal ($N = 18$) were collected if available and stored in the farm households, and were immediately sent to the laboratory for analysis. The total number of food products analyzed was 203. Most of the food products collected were industrialized products that were previously purchased by volunteers in supermarkets in nearby cities, and were available in their original packages (0.5-1.0 kg) in the households at the time of sampling. Upon arrival at the laboratory, all food samples were homogenized, finely milled and kept frozen at -20 °C until the moment of mycotoxin determination. During the visit to each farm, volunteers were instructed to collect their first morning urine (min. 10 mL) in the following day after food sample collection. After collection, urine samples were transported to the laboratory in a cooler with dry ice, and kept frozen at -20 °C until analysis. The total number of urine samples analyzed

in the two sampling periods was 162. Figure 5 presents an overview of the experimental protocol including sampling procedures adopted in the study.

Figure 5 - Flow chart describing the sampling procedures adopted in the study. N: number of collected samples



Source: Own authorship.

5.2.3 Food Consumption Questionnaires

At the time of food and urine sample collection, participants were instructed to complete a dietary recall questionnaire (RQ24h- ANEXX C) describing the consumption of foods in the 24 h before sample collection, including those that are usually considered of higher risk for mycotoxin contamination and regulated in Brazil (ANVISA, 2011). In addition, volunteers were instructed to complete a Food Consumption Frequency Questionnaire (ANEXX D). This questionnaire estimates the portion of high-risk foods (susceptible to mycotoxins occurrence) eaten by volunteers per unit of time. Consumption frequencies were estimated on the basis of the following terms: "Never", "Less than once a month", "1-3 times a month", "2-4 times a week", "Every day" and "2 times a day". Portions were estimated in grams, based on home measures that were common to the participants, such as cups, spoons, etc. (JAGER et al., 2013). RQ24h values were used to estimate the dietary intake of mycotoxins.

5.2.4 Reagents and solutions

Analytical grade reagent and Milli-Q (Millipore, Bedford, MA, USA) water, HPLC-grade acetonitrile and methanol (JT Baker, Xalostoc, Mexico) were used in all laboratory procedures. Mycotoxin standards (AFM_1, AFB_1, AFB_2, AFG_1, AFG_2, AFP_1, AFQ_1, OTA, FB_1, FB_2, ZEN, α-ZEL, β- ZEL, DON, DOM-1, 15-Ac-DON, T-2 and HT-2) were purchased from Sigma (Sigma, St Louis, MO, USA). Isotopically labeled standards (IS) of [$^{13}C_{17}$]-AFB_1 and [$^{13}C_{17}$]-AFM_1 (Sigma, St. Louis, MO, USA), [$^{13}C_{20}$]-OTA, [$^{13}C_{34}$]-FB_1, [$^{13}C_{18}$]-ZEN and [$^{13}C_{15}$]-DON (Biopure, Romer Labs, Tulln, Austria) were also used. Individual stock solutions were prepared in water/acetonitrile.

5.2.5 Preparation of food samples

Extraction of mycotoxins from food products was performed strictly following the procedures as described by Sulyok, Krska e Schuhmacher (2007), including minor modifications as proposed by Franco et al. (2018). Briefly, food samples were finely grinded and weighed (1.0 g) in duplicate 15-mL Falcon tubes. Then, 4 mL of the acetonitrile/water/acetic acid extraction solvent (80:20:0.1%, v/v/v) were added to the tubes, which were vortexed for 1 min, and homogenized in shaker (Tecnal) for 60 min. After that, they were vortexed again and kept under stirring for other 30 min. Samples were then centrifuged at 3,000 rpm (Centrifugal Quimis, Brazil) for 5 min. Supernatants were removed and filtered through 0.22 μm membrane filters (Millex, Millipore Corp.). An aliquot of 80

µL of the solution was transferred to a glass insert placed in a vial, and mixed with 20 µL of a mixture of IS previously prepared in water/acetonitrile (1:1, v/v) at the concentrations of 5 ng/mL for [$^{13}C_{17}$]-AFB$_1$, [$^{13}C_{20}$]-OTA, [$^{13}C_{24}$]-T-2 and [$^{13}C_{34}$]-FB$_1$; 250 ng/mL for [$^{13}C_{18}$]-ZEN and 500 ng/mL for [$^{13}C_{15}$]-DON. Final concentrations in each sample extract were 1 ng/mL for [$^{13}C_{17}$]-AFB$_1$, [$^{13}C_{20}$]-OTA, [$^{13}C_{24}$]-T-2, [$^{13}C_{34}$]-FB$_1$, 50 ng/mL for [$^{13}C_{18}$]-ZEN and 100 ng/mL for [$^{13}C_{15}$]-DON. Calibration curves were prepared with working solutions in water/acetonitrile (1:1, v/v) at levels ranging from 0.05-4.0 ng/mL of AFB$_1$, AFB$_2$, AFG$_1$, AFG$_2$, OTA, FB$_1$, FB$_2$, ZEN and T-2, 0.15-12.0 ng/mL of HT-2, and 0.37-30 ng/mL of DON.

5.2.6 Preparation of urine samples

The analysis of multiple mycotoxin residues and biomarkers in human urine was performed as described by Solfrizzo et al. (2011), with minor modifications. Prior to the extraction of mycotoxins in urine, samples were centrifuged at 3,000 x g for 5 min, to remove particulate matter and supernatants. After this procedure, 300 µL of β-glucuronidase/sulfatase was added to 6 mL of sample for the enzymatic deconjugation of mycotoxins. After this procedure, samples were incubated under static conditions at 37 °C overnight. Samples were then diluted in ultra-pure water (1:1, v/v), and 20 µL of an IS solution containing 3 µg/mL of [$^{13}C_{17}$]-AFM$_1$, [$^{13}C_{20}$]-OTA, [$^{13}C_{34}$]-FB$_1$, [$^{13}C_{24}$]-T-2 and [$^{13}C_{18}$]-ZEN and 6 µg/mL of [$^{13}C_{15}$]-DON was added to each sample. Immuno-affinity columns (Oasis HLB) and (Mycosep6in1) (Vicam, Watertown, MA, USA) were used for sample clean-up. The Oasis HBL column was attached under the Mycosep6in1 column (empty). The two stacked columns were positioned on a vacuum manifold with stopcocks. For the Oasis HBL column conditioning, 2 mL of MeOH was added, followed by 2 mL of ultra-pure water. The diluted urine sample previously prepared was passed through the two stacked columns, and then the two columns were separated and treated differently. Before elution on the Mycosep6in1 column began, the column was washed with 4 mL of water, and used the vacuum-dried for 15 seconds. The elution of mycotoxins started with the addition of 1.5 mL of MeOH until the first drop came out, then the stopcock was closed for 1 min and then opened until MeOH reached the top layer of the column, closing and adding another 1.5 mL of MeOH. After 1 min, the stopcock was opened and approximately 0.5 mL MeOH was eluted through the column by gravity and the stopcock was closed again for 1 min, then opened until elution of the MeOH was completed. Two mL of water was added

to the column, which was first eluted by gravity and subsequently vacuum-dried for complete elution.

The Oasis HLB column was washed with 1 mL of MeOH:water (2:8, v/v), then vacuum-dried for 15 seconds. The mycotoxins were eluted from the column with 1 mL MeOH:water (4:6, v/v) by gravity, and collected in the same vial containing the eluate from the Mycosep6in1 columns. The combined, final eluates were dried under N_2, re-suspended in methanol-water solution (1:9, v/v), filtered through 0.22 μm membrane filters (Millex, Millipore Corp.), and reserved for analysis of by liquid chromatography coupled to mass spectrometry (LC-MS/MS). For the calibration curves, working solutions were prepared in water/acetonitrile (9:1, v/v) at concentrations ranging from 0.01-0.3 ng/mL of AFM_1, AFP_1, AFQ_1, FB_1, FB_2, OTA, T-2, HT-2, ZEN, α-ZEL and β- ZEL, and 0.1-5.0 ng/mL for DON, DOM-1 and 15-Ac-DON.

5.2.7 Liquid chromatographic and mass spectrometry conditions

Final extracts from food and urine samples were injected into a Waters Acquity I-Class UPLC system (Waters, Milford, MA, USA) equipped with a BEH C_{18} column (2.1 × 50 mm, 1.7 μm) and coupled to a Xevo TQ-S mass spectrometer (Waters, Milford, MA, USA). For chromatographic separation, extracted samples and standards, 10 L of food samples or 5 L of urine samples were injected into the LC-MS/MS system. The column was kept at 40°C during the analyses, and samples were maintained at 15°C. The mobile phase was composed by water (eluent A) and acetonitrile (eluent B), both containing 0.1% of formic acid. For elution of injected samples, percentage of eluent A was kept at 95% for 0.5 min. After this period, percentage of eluent B was linearly raised to 25% over 4.5 min (5.0 min). Then, eluent B was increased to 90% over 0.5 min, followed by a hold time of 0.25 min (5.5 min). After that, percentage of eluent B was reduced to 5% over 0.5 min (6.0 min), and the column re-equilibrated to the initial conditions for 0.5 min. Total chromatographic run time was 6.5 min, and the mobile phase flow rate was maintained at 0.5 mL/min. The mass spectrometer was operated in MRM mode using electrospray ionization in either positive or negative ion mode. Mass spectrometry parameters were as follows: capillary voltage: 0.75 kV; source temperature: 150°C; desolvation temperature: 500°C, desolvation gas flow: 800 L/h; cone gas flow: 150 L/h. Cone voltage, collision energy, and MRM transitions (major precursor ion > fragment ion) were manually optimized for individual mycotoxins. Data collection and processing was performed using software MassLynx version 4.1.

5.2.8 Performance of the analytical methods

The analytical method for foods was previously validated for corn products (FRANCO et al., 2018). Thus, the performances of the analytical methods were evaluated using blank samples of rice, bean and wheat flour, as well as urine. Parameters evaluated included limits of detection (LOD) and quantification (LOQ) (calculated based on signal-to-noise ratios of 3:1 and 10:1, respectively, of peaks of confirmatory MRM transitions); apparent recovery (RA); linearity; signal suppression/enhancement (SSE) due to matrix effects; and extraction recovery (RE). All these parameters were determined based on calibration curves constructed from the analytical data obtained in samples spiked before extraction, spiked after extraction, and standards diluted in solvent, with the addition of the IS working solution in all prepared samples.

Spiked food samples were prepared exactly as described by Franco et al. (2018) in triplicate tubes containing blank samples (1.0 g) at concentrations ranging from 1.25-20 µg/kg for AFB_1, AFB_2, AFG_1, AFG_2, OTA, FB_1, FB_2, T-2, HT-2 and ZEN, and 18.7-150 µg/kg for DON. The spiked samples were submitted to the same extraction procedures as described for samples collected in the farms. For the matrix-matched calibration curves, spiked extracts were prepared in triplicate with blank samples (1.0 g) of each type of product at concentrations ranging from 0.25-4.0 ng/mL for AFB_1, AFB_2, AFG_1, AFG_2, OTA, FB_1, FB_2, T-2, HT-2 and ZEN, and 3.7-30 ng/ mL for DON. The same levels were used to prepare standard solutions in water/acetonitrile (9:1, v/v). Eighty µL of each level of final extracts from spiked samples, spiked extracts and liquid standards were combined with 20 µL of the IS working solution in glass inserts inside amber vials and stored at -20 ˚C until analysis by LC-MS/MS.

Urine fortified samples were prepared by adding appropriate volumes of standard working solutions to triplicate tubes containing blank samples (urine:ultrapure water, 1:1, v/v) before the elution, to achieve concentrations ranging from 0.01-0.3 ng/mL for AFM_1, AFP_1, AFQ_1, FB_1, FB_2, OTA, T-2, HT-2, ZEN, α-ZEL and β- ZEL, and 0.1-5.0 ng/mL for DOM-1, DON, 15-Ac-DON. Clean-up and elution procedures were carried out as described in section 2.6. Standard solutions in water/acetonitrile (9:1, v/v) and matrix-matched calibration curves were prepared by spiking triplicate blank extracts with appropriate volumes of the mycotoxin working solutions to reach the same concentrations as described for spiked samples. For all urine samples, 20 µL of the IS solution was added before elution to 80 µL of each level from fortified samples, spiked and liquid standards. Values for RA

(Eq. 1), SSE (Eq. 2) and RE (Eq. 3) for the analytical methods for foods and urine were calculated as proposed by Sulyok et al. (2006) and Varga et al. (2012).

RA (%) = 100* slope spiked sample /slope standard diluted in solvent (Eq. 1)

SSE (%) = 100* slope spiked extract /slope standard diluted in solvent (Eq. 2)

RE (%) = 100*RA / SSE (Eq. 3)

5.2.9 Creatinine analysis in human urine

Creatinine analysis was performed in urine samples using a commercial kit (Bioplus - Bio 200), with two-point kinetic assay based on the Jaffe reaction principle (VASILIADES, 1976). The concentration of creatinine in each urine sample was used to correct differences in dilution between individuals and excretion rates, and the results were expressed in ng mycotoxin / mg creatinine.

5.2.10 Exposure assessment (probable daily intakes estimates) and risk characterization

In the present study, exposure assessment was determined based on estimated intake through food (indirect approach) and through urine biomarkers (direct approach). Eq. 4 shows the calculation of PDI through food, according to Assunção et al. (2015).

$$PDI = \frac{\text{Occurrence} * \text{Consumption}}{\text{b.w.} * 1000} \quad \text{(Eq. 4)}$$

- PDI = Probably Daily Intake (μg/kg b.w. /day);
- Occurrence = mycotoxin content (μg/kg) determined in food analysis;
- b.w. = body weight (kg) reported by volunteers;
- Consumption = reported consumption (g) of food on the previous day.

The methodology described by Turner et al. (2010) was used to calculate the PDI through urinary biomarkers data (Eq. 5).

$$PDI = \frac{\text{Occurrence} * V}{ER * \text{b.w.} * 1000} \quad \text{(Eq. 5)}$$

- PDI = Probably Daily Intake (µg/kg b.w. /day);
- Occurrence = mycotoxin content (ng/mL) determined in urine analysis;
- V = daily urine production of adults, assumed to be 1,500 mL (TURNER et al., 2010);
- b.w. = body weight (kg) reported by volunteers;
- ER = urinary excretion ratio of AFM_1 for women: 1.5% (ZHU et al., 1987);
  = urinary excretion ratio of AFM_1 for men: 1.7% (ZHU et al., 1987);
  = urinary excretion ratio of DON for women: 72% (VIDAL et al., 2018);
  = urinary excretion ratio of DON for men: 50% (VIDAL et al., 2018);
  = urinary excretion ratio of OTA: 50% (SCHLATTER; STUDERROHR; RASONYI, 1996);
  = urinary excretion ratio of FB_1: 0.5% (RILEY et al., 2012);
  = urinary excretion ratio of ZEN: 36.8% (GAMBACORTA et al., 2013).

Risk characterization was performed comparing PDI values with dose reference values of tolerable daily intake (TDI) for OTA (0.016 µg/kg b.w./day) (FAO/WHO, 2007), FBs (2 µg/kg b.w. /day) (FAO/WHO, 2011a) DON (1.0 µg/kg b.w. /day) (FAO/WHO, 2011b) and ZEN (0.25 µg/kg b.w. /day) (EFSA, 2016). Comparisons were performed using hazard coefficients (HQ) ratio between exposure and a reference dose) as referred at Eq. 6. HQ <1 indicated tolerable exposure and a HQ >1 ratio indicated a non-tolerable exposure level (BORG et al., 2013; EFSA, 2013).

$$HQ = \frac{PDI}{TDI} \qquad \text{(Eq. 6)}$$

- HQ = Hazard Quotient;
- PDI = Probable Daily Intake (µg/kg b.w. /day);
- TDI = Tolerable Daily Intake (µg/kg b.w. /day).

Considering the carcinogenic potential of aflatoxins, the Margin of Exposure (MoE) was calculated for exposure to this toxin (Eq. 7) as a ratio of the Benchmark Dose Lower Confidence Limit ($BMDL_{10}$) and the level of exposure (PDI). MoE indicates the risk level, with MoE ≥ 10,000 being of low public health concern, and MoE < 10,000 being of high public health concern (EFSA, 2013). For aflatoxins, the $BMDL_{10}$ value was in accordance with Benford, Leblanc e Setzer (2010).

$$MoE = \frac{BMDL_{10}}{PDI} \quad \text{(Eq. 7)}$$

- MoE = Margin of Exposure;
- $BMDL_{10}$ = Benchmark Dose Lower Confidence Limit (0.25 μg/kg b.w./day);
- PDI = Probably Daily Intake (μg/kg b.w. /day).

5.2.11 Statistical analysis

Data were statistically analyzed using an IBM SPSS Statistics 23 software. The differences in the PDI of each mycotoxin evaluated in the two sampling periods and places of sampling were evaluated using a non-parametric Mann-Whitney test, considering a 95% confidence interval and $P<0.05$. For the purpose of data analysis, only positives samples (mycotoxin concentration above the LOQ) were considered.

5.3 Results

5.3.1 Performance of the analytical methods

Table 14 presents the performance parameters of the analytical method for determination of mycotoxins in rice, bean, and wheat flour samples. The LOD and LOQ values for individual mycotoxins ranged from 0.12 to 6.1 μg/kg and 0.3 to 18.8 μg/kg, respectively. R_A, SSE and R_E values for mycotoxins ranged from 59 to 127%, 63 to 113% and 76 to 143%, respectively. The same parameters were determined for the analytical method used for determination of mycotoxin biomarkers in urine samples, and the results are presented in Table 15. LODs and LOQs values ranged from 0.001 to 0.633 ng/mL and 0.003 to 2.000 ng/mL, respectively. The determined R_A, SSE and R_E values ranged from 56 to 93%, 62 to 103% and 80 to 113%, respectively.

Table 14 - Method performance parameters for determination of mycotoxins in samples of food (rice, bean and wheat flour)

continue

Mycotoxin	RT (min)	Concentration range (μg/kg)[a]	R_A range (%)	SSE range (%)	R_E range (%)	LOD (μg/kg)	LOQ (μg/kg)
AFB_1	4.80	1.25-20	105-110	79-99	109-140	0.4-0.5	0.8-1.0
AFB_2	4.50	1.25-20	64-106	68-103	94-102	0.4-0.5	0.8-1.0

Table 14 - Method performance parameters for determination of mycotoxins in samples of food (rice, bean and wheat flour)

conclusion

Mycotoxin	RT (min)	Concentration range (μg/kg)[a]	R_A range (%)	SSE range (%)	R_E range (%)	LOD (μg/kg)	LOQ (μg/kg)
AFG_1	4.46	1.25-20	89-107	95-113	94-98	0.4-0.5	0.8-1.0
AFG_2	4.18	1.25-25	64- 103	84-97	76-108	0.4-0.6	0.9-1.0
DON	1.98	18.7-150	101-103	63-109	93-105	5.8-6.1	18.5-18.8
OTA	5.99	1.25-20	84-106	92-99	92-109	05.-0.7	1.0-1.1
HT-2	5.50	15-60	97-105	99	98-106	6.0-6.5	15.0-16.0
T-2	5.91	1.25-20	105-115	97-98	105-108	0.3-0.6	1.0-1.4
FB_2	3.74	2.5-25	91-101	90-99	101-102	07-0.9	2.0-2.5
FB_1	5.40	2.5-25	59-109	98-102	97-111	0.9-1.0	2.0-2.8
ZEN	5.98	0.25-20	97-127	89-98	95-143	0.12-0.23	0.3-0.6

Source: Own authorship. [a] Expressed as minimum-maximum values observed between the food products; RT: retention time; R_A: apparent recovery; SSE: signal suppression/enhancement; R_E: extraction recovery; LOD: limit of detection; LOQ: limit of quantification; AF: aflatoxin. DON: deoxynivalenol. OTA: ochratoxin A. FB: fumonisin. ZEN: zearalenone.

Table 15 - Method performance parameters for determination of mycotoxins in samples of urine

Mycotoxin	RT (min)	Concentration range (μg/kg)	R_A (%)	SSE (%)	R_E (%)	LOD (μg/kg)	LOQ (μg/kg)
AFP_1	4.46	0.05-0.8	67.5	82.1	82.2	0.013	0.050
AFM_1	4.03	0.01-0.27	80.9	101.0	80.2	0.001	0.004
AFQ_1	4.50	0.05-0.8	50.5	62.3	81.1	0.020	0.067
DON	1.98	0.10-5.0	75.5	90.2	83.7	0.333	1.223
DOM	2.58	0.10-5.0	90.4	79.9	113.1	0.200	0.310
15-Ac-DON	3.38	0.10-5.0	70.1	87	80.6	0.633	2.000
OTA	5.99	0.01-0.27	57.0	71.0	80.3	0.005	0.017
HT-2	5.50	0.05-0.8	56.1	72.0	79.6	0.024	0.075
T-2	5.91	0.01-0.27	59.2	74.8	80.1	0.013	0.040
FB_1	5.40	0.01-0.27	81.1	99.9	81.2	0.003	0.007
FB_2	3.74	0.01-0.27	82.4	102.5	80.4	0.001	0.013
ZEN	5.98	0.01-0.27	92.6	99.9	92.7	0.001	0.003
α -ZEL	5.53	0.05-0.8	91.9	89.6	102.6	0.053	0.183
β -ZEL	5.76	0.05-0.8	90.4	91	99.3	0.060	0.200

Source: Own authorship. RT: retention time; R_A: apparent recovery; SSE: signal suppression/enhancement; R_E: extraction recovery; LOD: limit of detection; LOQ: limit of quantification; AF: aflatoxin. DON:

deoxynivalenol. OTA: ochratoxin A; FB: fumonisin. ZEN: zearalenone; DOM: deepoxydeoxynivalenol; α-ZEL: α-zearalenol; β-ZEL: β-zearalenol; 15-Ac-DON: 15- acetyl-DON.

5.3.2 Occurrence of mycotoxins in food products

Table 16 presents the mycotoxin levels in food samples collected in households of small-scale farms from the states of São Paulo and Santa Catarina. All types of foods showed positive samples containing at least one type of mycotoxin above LOQ, comprising 38% of rice (*N*=66), 12% of bean (*N*=59), 97% of wheat flour (*N*=39), 100% of corn flour (*N*=21), and 94% of corn meal (*N*=18) samples. AFs were found in two samples of rice (3%), one sample of bean (2%), and two samples of corn flour (10%), with the highest median of total AF observed in rice samples (2.9 μg/kg). DON was found in all types of foods, with median values of 13.2 μg/kg (rice), 51.3 μg/kg (bean), 408.2 μg/kg (wheat flour), 56.7 μg/kg (corn flour), and 51.8 μg/kg (corn meal). OTA was only found in three samples (8%) of wheat flour with a median value of 0.9 μg/kg. T-2 toxin was found in only one sample of rice (1.5%) containing 1.0 μg/kg. However, HT-2 was not detected in any sample evaluated. FBs were also detected in all types of food, except for bean samples, with median levels (FB_1 + FB_2) of 1.5, 14.6, 131.6 and 131.9 μg/kg in rice, wheat flour, corn flour, and corn meal, respectively. ZEN was detected in 18 (27%) samples of rice, 4 (19%) of corn flour and a single sample of corn meal (6%), at median values of 4.9, 98.6 and 10.7 μg/kg, respectively.

Table 16 - Occurrence of mycotoxins in food samples collected in households of small-scale farms from the states of São Paulo and Santa Catarina, Brazil

continue

	AFB_1	AFB_2	AFG_1	AFG_2	∑AF	DON	OTA	HT-2	T-2	FB_1	FB_2	∑FB	ZEN
Rice (*N* = 66):													
n (%)	1 (1.5)	0	0	1 (1.5)	2 (3)	8 (12)	0	0	1 (1.5)	0	1 (1.5)	1 (1.5)	18 (27)
Range (µg/kg)	3.9	<LOQ	<LOQ	2.0	2.0-3.9	7.0-171.7	<LOQ	<LOQ	1.0	<LOQ	1.5	1.5	1.7-230.0[1]
Median (µg/kg)	-	-	-	-	2.9	13.2	-	-	-	-	-	-	4.9
Bean (*N* = 59):													
n (%)	0	0	0	1 (2)	1 (2)	6 (10)	0	0	0	0	0	0	0
Range (µg/kg)	<LOQ	<LOQ	<LOQ	2.0	2.0	46.5-60.2	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Median (µg/kg)	-	-	-	-	-	51.3	-	-	-	-	-	-	-
Wheat flour (*N* = 39):													
n (%)	0	0	0	0	0	38 (97)	3 (8)	0	0	3 (8)	4 (10)	5 (13)	0
Range (µg/kg)	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	27.8-2203.8[2]	0.6-1.4	<LOQ	<LOQ	3.2-43.6	11.5-272.4	3.2-316.0	<LOQ
Median (µg/kg)	-	-	-	-	-	408.2	0.9	-	-	25	101.5	14.6	-
Corn flour (*N* = 21):													
n (%)	1 (5)	0	1 (5)	0	2 (10)	9 (43)	0	0	0	21 (100)	9 (43)	21 (100)	4 (19)
Range (µg/kg)	3.6	<LOQ	1.3	<LOQ	1.3-3.6	43.9-78.6	<LOQ	<LOQ	<LOQ	6.8-883.0	89.8-630.5	6.8-1513.45[3]	8.7-508.88[4]
Median (µg/kg)	-	-	-	-	2.48	56.7	-	-	-	115.6	235.0	131.6	98.6

Table 16 - Occurrence of mycotoxins in food samples collected in households of small-scale farms from the states of São Paulo and Santa Catarina, Brazil

conclusion

	AFB_1	AFB_2	AFG_1	AFG_2	∑AF	DON	OTA	HT-2	T-2	FB_1	FB_2	∑FB	ZEN
Corn meal (*n* = 18):													
n (%)	0	0	0	0	0	2 (11)	0	0	0	16 (89)	9 (50)	17 (94)	1 (6)
Range (µg/kg)	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	43.0-60.6	<LOQ	<LOQ	<LOQ	2.9-796.2	5.9-496.5	2.9-1169.2	10.7
Median (µg/kg)	-	-	-	-	-	51.8	-	-	-	122.2	87.0	131.9	-

Source: Own authorship. [1] One sample with concentration above the maximum permitted level (MPL) for ZEN in rice (100 µg/kg) in Brazil (Agência Nacional de Vigilância Sanitária, 2014).

[2] Five samples with concentration above the MPL for DON in wheat flour (750 µg/kg) in Brazil (Agência Nacional de Vigilância Sanitária, 2014).

[3,4] One sample with concentration above the MPL for total FB and two samples above the MPL for ZEN in corn flour (1,500 and 150 µg/kg, respectively) in Brazil (Agência Nacional de Vigilância Sanitária, 2014).

n: Number of samples with concentrations above the limit of quantification (LOQ), see Table 14 for LOQ of each mycotoxin.

AF: aflatoxin; DON: deoxynivalenol; OTA: ochratoxin A; FB: fumonisin; ZEN: zearalenone; NA: Not applicable (no regulations applied in Brazil)

5.3.3 Estimation of mycotoxin exposure through food data

Total PDI estimates through food are presented in Table 17. PDI mean values were calculated for mycotoxins with reference values available for AF, DON, OTA, FB and ZEN, and based on the intake of foods (Supplementary material A – ANNEX F), the body weight of the volunteers (Supplementary material B – ANNEX G) and the mycotoxin occurrence data. For total AF, only rice and bean contributed for the PDI at mean values of 0.013 ± 0.007 and 0.007 μg/kg b.w./day, respectively. All types of foods analyzed contributed for a PDI of DON at mean values ranging from 0.069 ± 0.032 to 1.002 ± 0.772 μg/kg b.w./day. The PDI of total FB contributed by wheat flour, corn flour and corn meal ranged from 0.119 ± 0.133 to 0.321 ± 0.312 μg/kg b.w./day. Regarding ZEN, only rice and corn flour contributed for mean PDI values of 0.156 ± 0.310 and 0.013 ± 0.001 μg/kg b.w./day.

Table 17 - Probable daily intake (PDI) of mycotoxins based on their levels in foods consumed by volunteers in small-scale farms from the states of São Paulo and Santa Catarina, Brazil[1]

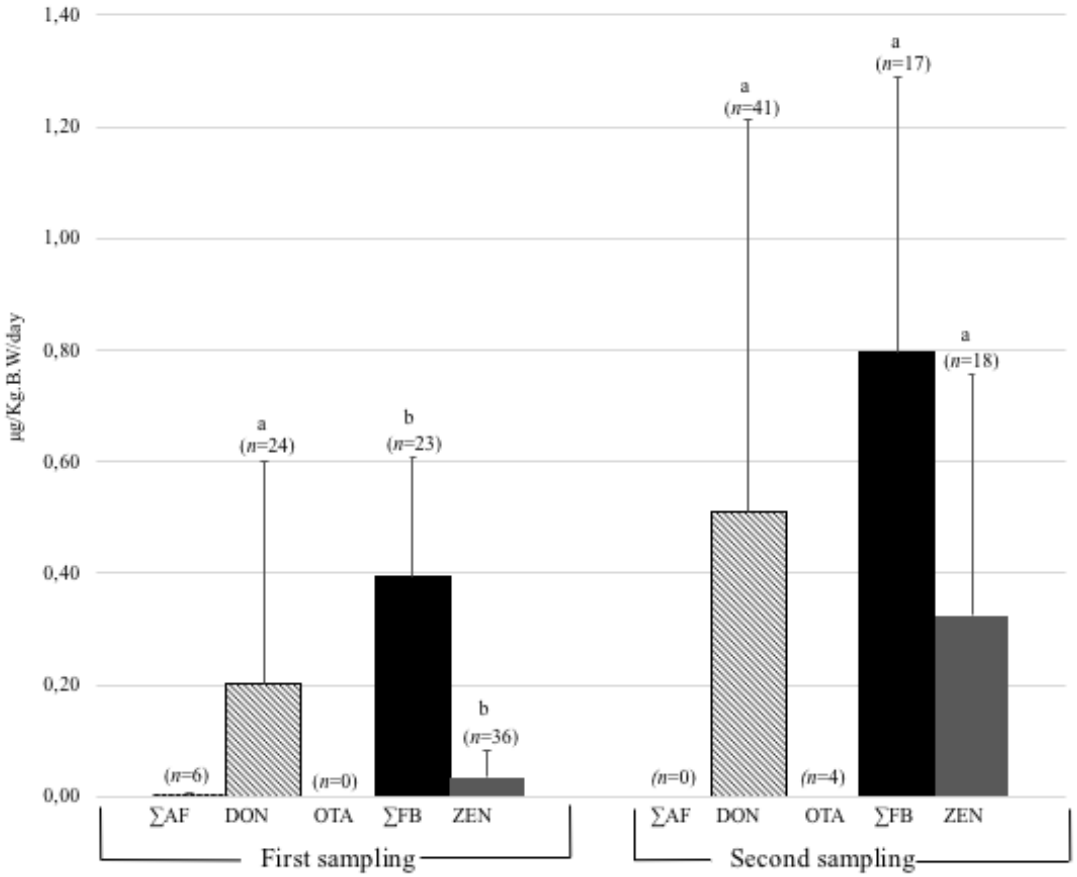
Mycotoxin	PDI (μg/kg body weight/day)[2]				
	Rice	Bean	Wheat flour	Corn flour	Corn meal
AFB_1	0.018 ± 0.001	0	0	0	0
AFG_2	0.007 ± 0.001	0.007 ± 0.00	0	0	0
∑AF	0.013 ± 0.007	0.007 ± 0.00	0	0	0
DON	0.069 ± 0.073^{c}	0.086 ± 0.044^{b,c}	1.002 ± 0.772^{a}	0.124 ± 0.070^{b}	0.069 ± 0.032^{b,c}
OTA	0	0	0	0	0
FB_1	0	0	0.032 ± 0.019	0.257 ± 0.234	0.311 ± 0.316
FB_2	0	0	0.177 ± 0.151	0.475 ± 0.153	0.351 ± 0.344
∑FB	0	0	0.119 ± 0.133^{a}	0.305 ± 0.235^{a}	0.321 ± 0.312^{a}
ZEN	0.156 ± 0.310^{a}	0	0	0.013 ± 0.001^{b}	0

Source: Own authorship. [1] Results are expressed as mean ± standard deviation of individual PDI values, calculated considering food samples with concentrations above the limit of quantification (LOQ) and food consumption data from each volunteer (Supplementary B – ANNEX G).
[2] Calculated as follows: PDI = (occurrence rate x consumption) / (body weight x 1000).
[a-c] In the same row, means followed by different superscript letters differ significantly (P<0.05).
AF: aflatoxin; DON: deoxynivalenol; OTA: ochratoxin A; FB: fumonisin; ZEN: zearalenone.

Individual PDI estimates through contaminated food products in the two sampling periods are presented in Figure 6. Statistical differences (P<0.05) between the first and the second sampling were observed in the PDI values of FBs and ZEN, both being higher in the second collection. For FBs, PDI values varied from 0.20 ± 0.21 μg/kg b.w./day to 0.52 ± 0.49 μg/kg b.w./day, in the first and second sampling, respectively. For ZEN, PDI

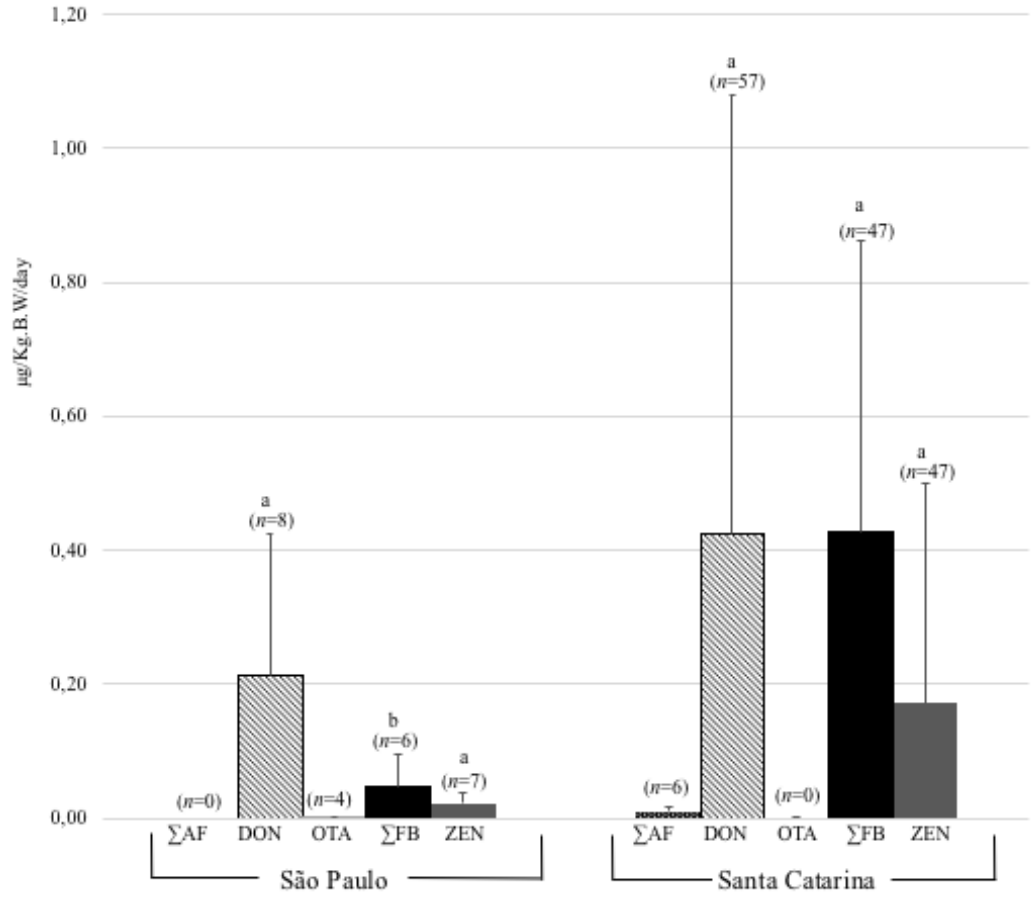
varied from 0.04 ± 0.05 to 0.32 ± 0.43 μg/kg b.w./day in the first and second sampling, respectively. PDI estimates through contaminated foods collected in small-scale frmas from different states of Brazil are presented in Figure 7. Overall the highest values were found in the state of Santa Catarina, although no significant differences were found except for total FB, which was significantly lower (P<0.05) in São Paulo.

Figure 6 - Estimated dietary intake of mycotoxins in positive samples (n) based on consumed food levels in two sampling periods. Results are expressed as mean ± SD. For the same type of mycotoxin, bars with different superscript letters differ significantly (P<0.05). Total AF: sum of aflatoxins B_1, B_2, G_1 and G_2; DON: deoxynivalenol (DON); OTA: ochratoxin A; Total FB: sum of fumonisins B_1 and B_2; ZEN: zearalenone



Source: Own authorship.

Figure 7 - Estimated dietary intake of mycotoxins in positive samples (n) based on consumed food levels in the states of Santa Catarina and São Paulo, Brazil. Results are expressed as mean ± SD for the same type of mycotoxin, bars with different superscript letters differ significantly (P<0.05). Total AF: sum of aflatoxins B_1, B_2, G_1 and G_2; DON: deoxynivalenol (DON); OTA: ochratoxin A; Total FB: sum of fumonisins B_1 and B_2; ZEN: zearalenone.



Source: Own authorship.

5.3.4 Occurrence of mycotoxin biomarkers in human urine

Table 18 presents the levels of mycotoxins biomarkers in positive samples (above LOQs) of urine (*N*=162) collected in small-scale farms from the states of São Paulo and Santa Catarina. DON was the most frequent mycotoxin found in urine, with 88% of positive samples at levels ranging from 0.62 to 72,439 ng/mg creatinine (median: 12.0 ng/mg creatinine). However, DOM-1 was detected in only one sample (0.6%) at 7.3 ng/mg creatinine. Concerning AFs, only AFM_1 and AFP_1 were detected in 17% of urine samples, at median levels of 0.02 ng/mg of creatinine. OTA and ZEN were detected in 27% and 7% of samples, respectively, with the same median value for both mycotoxins (0.02 ng/mg creatinine). FB_1 was present in 23% of the samples at 0.06 ng/mg creatinine. AFQ_1, 15-Ac-DON, T-2, HT-2, FB_2, α-ZEL and β- ZEL were not detected in any urine sample.

Table 18 - Mycotoxin levels in urine samples (N=162) from volunteers in small-scale farms from the states of São Paulo and Santa Catarina, Brazil

Mycotoxin	*n*	%	Range (ng/mg de creatinine)	Median (ng/mg de creatinine)
AFM_1	20	12	0.0005 - 0.64	0.02
AFP_1	9	6	0.01 - 0.08	0.02
AFQ_1	0	0	<LOQ	<LOQ
∑AF	27	17	0.0005 - 0.72	0.02
DON	143	88	0.62 - 72439.28	12.0
DOM-1	1	0.6	7.3	-
15-Ac-DON	0	0	<LOQ	<LOQ
OTA	44	27	0.01 - 11.71	0.02
HT-2	0	0	<LOQ	<LOQ
T-2	0	0	<LOQ	<LOQ
FB_1	37	23	0.01 - 0.29	0.04
FB_2	0	0	<LOQ	<LOQ
∑FB	37	23	0.01 - 0.29	0.04
ZEN	12	7	0.01 - 0.77	0.02
α-ZEL	0	0	<LOQ	<LOQ
β-ZEL	0	0	<LOQ	<LOQ

Source: Own authorship. *n*: Number of samples showing concentrations above the limit of quantification (LOQ), see Table 15 for LOQ of each mycotoxin.
AF: Aflatoxin; DON: deoxynivalenol; OTA: ochratoxin A; FB: fumonisin; ZEN: zearalenone; α-ZEL: α-zearalenol; β-ZEL: β-zearalenol; 15-Ac-DON: 15- acetyl-DON.

5.3.5 Estimation of mycotoxin exposure through urinary levels

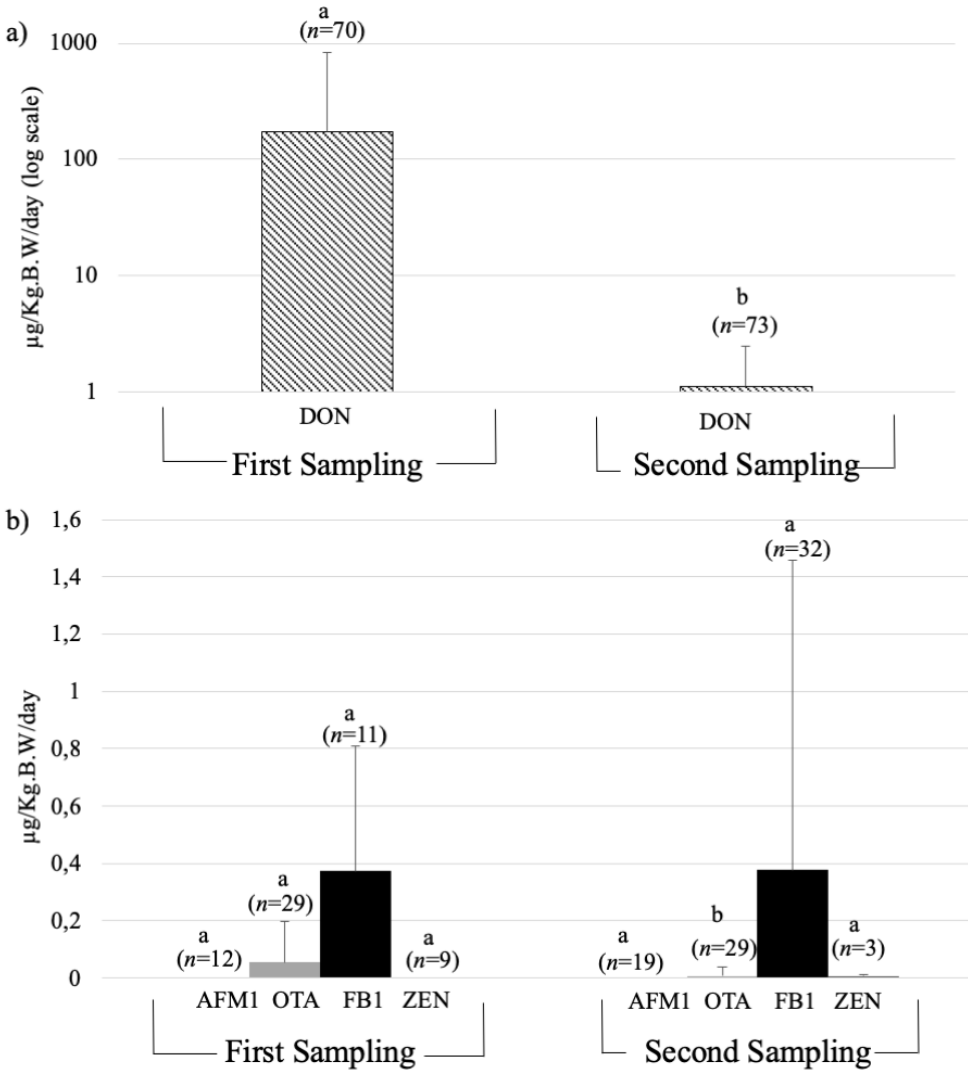
PDI estimates through urinary levels of mycotoxins biomarker are shown in Table 19. The PDI values were calculated according to previously described urinary excretion rates for AFs, DON, OTA, FBs and ZEN. In our study, the mean PDI ranged from 0.001 ± 0.002 to 84.914 ± 469.333 μg/kg b.w./day for AFM_1 and DON, respectively. When comparing the differences between the first and second sampling (Figure 8), urinary PDI for DON and OTA were greatly reduced from 172.31 ± 661.92 to 1.11 ± 1.35 μg/kg b.w./day, and from 0.0538 ± 0.1443 to 0.0081 ± 0.290 μg/kg b.w./day, respectively. However, the mean PDI for FB_1 did not vary (P>0.05) between the two sampling periods. Mean PDI values for DON, OTA, and FB_1 in the state of Santa Catarina were higher than the values found in the state of São Paulo, as presented in Figure 9, although differences were significant (P<0.05) only for DON.

Table 19 - Probable daily intake (PDI) of mycotoxins based on respective urinary levels of volunteers in small-scale farms from the states of São Paulo and Santa Catarina, Brazil[1]

Mycotoxin	UER		PDI (μg/kg body weight/day)[2]
	%	Reference	
AFM_1	Women: 1.5; Men: 1.7	(Zhu et al., 1987)	0.001 ± 0.002
DON	Women: 72; Men: 50	(Vidal et al., 2018)	84.914 ± 469.333
OTA	50	(Schlatter et al., 1996)	0.031 ± 0.106
FB_1	0.5	(Riley et al., 2012)	0.377 ± 0.951
ZEN	36.8	(Gambacorta et al., 2013)	0.002 ± 0.004

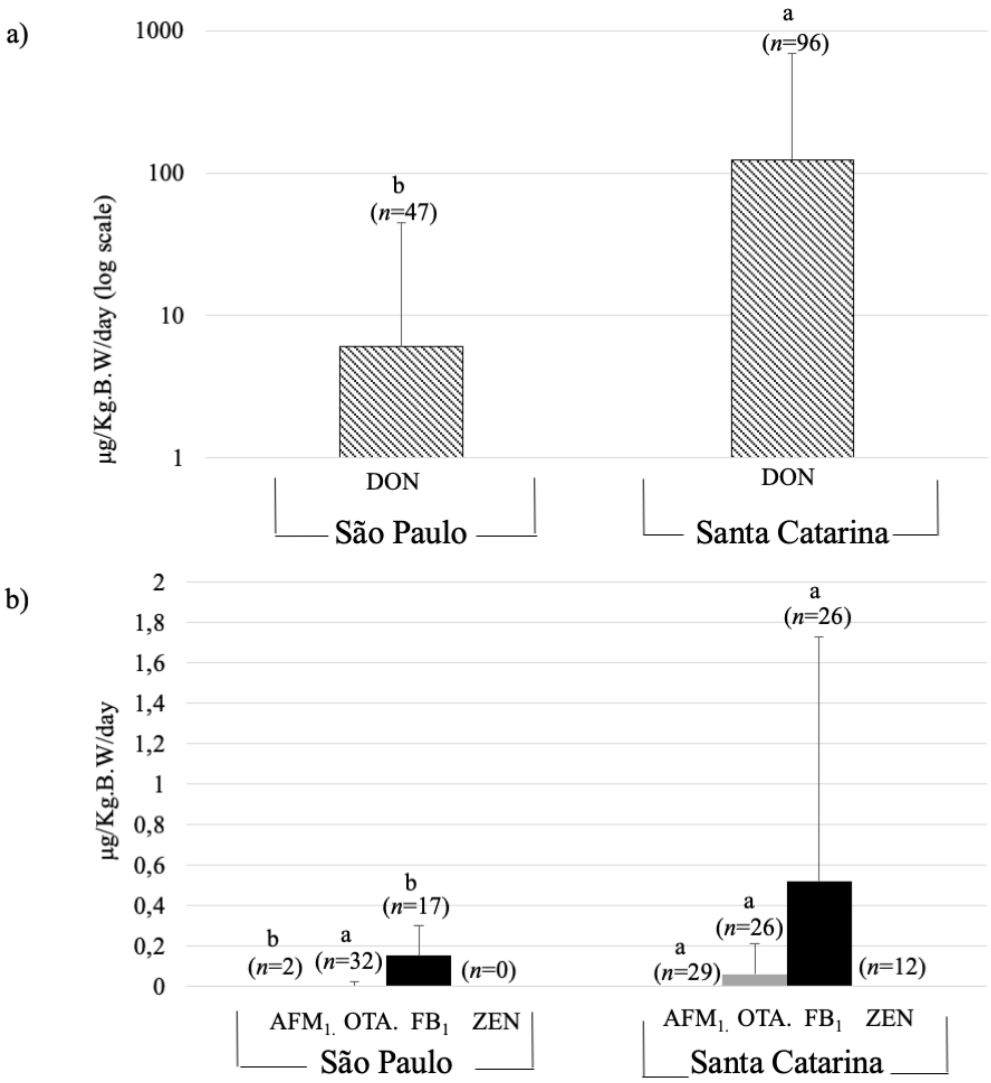
Source: Own authorship. [1] Results are expressed as mean ± standard deviation of individual PDI values, calculated considering urine samples with concentrations above the limit of quantification (LOQ).
[2] Calculated as follows: PDI = (occurrence rate x daily urine production) / (ER x body weight x 1000).
UER: Urinary excretion ratio; AFM_1: aflatoxin M_1; DON: deoxynivalenol; OTA: ochratoxin A; FB_1: fumonisin B_1; ZEN: zearalenone.

Figure 8 - Estimated dietary intakes based on urinary levels in positive samples (n), in two sampling periods, for: a) DON (log scale); b) AFM_1, OTA, FB_1 and ZEN. Results are expressed as mean ± SD. For the same type of mycotoxin, bars with different superscript letters differ



Source: Own authorship.

Figure 9 - Estimated dietary intakes based on urinary levels in positive samples (n) in the states of Santa Catarina and São Paulo, Brazil, for: a) DON (log scale); b) AFM1, OTA, FB1 and ZEN. Results are expressed as mean ± SD. For the same type of mycotoxin, bars with different superscript letters differ significantly (P<0.05). Total AF: sum of aflatoxins B_1, B_2, G1 and G_2; DON: deoxynivalenol (DON); OTA: ochratoxin A; Total FB: sum of fumonisins B_1 and B_2; ZEN: zearalenone



Source: Own authorship.

5.3.6 Risk characterization

Table 20 presents the HQ values derived from the PDI calculated through food data (indirect approach) and urinary biomarkers of mycotoxins (direct approach). For the indirect approach, HQ values greater than 1 was observed only for ZEN in the second sampling (1.07). For the direct approach, HQ values greater than 1 were observed for DON in both sampling periods (172.31 and 1.13), and for OTA in the first sampling (3.36). The MoE and MoET values for AFs related to their respective PDIs obtained in food and urine samples are presents in Table 21. Food samples had concentrations above LOQs for AFB_1 and AFG_2 only in the first sampling. For both types of AF, MoE values

were much lower than the reference level (10,000) in the first sampling, although no calculation was possible in the second sampling because at that time no sample had concentrations above LOQs for AFB_1 and AFG_2. Regarding the urine samples, MoE values for AFM_1 were 258.29 and 458.71 in the first and second samplings, respectively.

Table 20 - Risk characterization of mycotoxins through determination of Hazard Quotient (HQ) based on the occurrence data on food products and urine collected in small-scale farms from the states of São Paulo and Santa Catarina, Brazil

	HQ[1]	
Mycotoxin	First sampling	Second sampling
Food products[2]:		
DON	0.22	0.58
OTA	0.00	0.04
∑FB	0.11	0.29
ZEN	0.10	1.07*
Urine[2]:		
DON	172.31*	1.13*
OTA	3.36*	0.55
∑FB	0.19	0.21
ZEN	0.00	0.04

Source: Own authorship. * Indicates a non-tolerable risk (HQ > 1).
[1] Calculated as follows: HQ = Probable daily intake / reference values.
[2] Relative to samples containing mycotoxin levels above the limit of quantification (LOQ), see Tables 14 and 2 for LOQ of each mycotoxin in food products and urine, respectively.
DON: deoxynivalenol; OTA: ochratoxin A; FB: fumonisins; ZEN: zearalenone.

Table 21 - Risk characterization of aflatoxins through the determination of the Margin of Exposure (MoE) based on the occurrence data on food products and urine collected in small-scale farms from the states of São Paulo and Santa Catarina, Brazil

	MoE[1]	
Mycotoxin	First sampling	Second sampling
Food products[2]:		
AFB_1	0.019*	ND
AFG_2	0.007*	ND
Urine[2]:		
AFM_1	258.29*	458.71*
	MoET[3]	
Food products[2]:		
∑AF	0.005*	ND

Source: Own authorship. * Indicates high concern for public health (MoE < 10,000).
[1] Calculated as follows: MoE = Benchmark Dose Lower Confidence Limit (0.00025 mg/kg bw/day) / exposure data.

[2] Relative to samples containing mycotoxin levels above the limit of quantification (LOQ), see Tables 14 and 15 for LOQ of each mycotoxin in food products and urine, respectively.
[3] Calculated as follows: $MoET = 1 / [(1 / MoE_{AFB_1}) + (1 / MoE_{AFB_2}) + (1 / MoE_{AFG_1}) + (1 / MoE_{AFM_1})]$.
MoET: Combined Margin of Exposure. ND: Not determined (no sample with concentrations above LOQ).

5.4 Discussion and Conclusion

5.4.1 Performance of the analytical methods

The LOQ values for individual mycotoxins in food products (Table 14) were much lower than the MPLs for mycotoxins in foods determined by the Brazilian regulations (ANVISA, 2011). In order to assess the capacity of the analytical method to compensate the matrix effects, R_A, SSE and R_E values for mycotoxins were determined in rice, bean, and wheat flour samples with the addition of IS to samples (VARGA et al., 2012). R_E values were high for all mycotoxins evaluated (76–140%), which fulfil the requirements described by the European Commission (2006). The analytical method for determination of urinary biomarkers also showed high R_E values and suitable sensitivity for quantification of all mycotoxins evaluated, since LOQs ranged from 0.003 to 2.0 ng/mL (Table 15).

5.4.2 Mycotoxin contents in food products

All types of foods evaluated in the present study had samples containing quantifiable levels of at least one type of mycotoxin (Table 16). However, HT-2 was not detected in any sample analyzed, and T-2 was found in only one sample of rice at low concentration (1.0 μg/kg). The levels of AFB_1 and OTA found in rice were similar to those reported by Almeida et al. (2012), who observed mean concentrations of 2.49 and 0.64 μg/kg for total AF and OTA, respectively. AFs are the most frequently reported mycotoxins in rice, with AFB_1 values ranging from 0.1 to 308.0 μg/kg in India (REDDY; REDDY; MURALIDHARAN, 2009), and mean levels of 4.6 μg/kg for total AF in Pakistan (LUTFULLAH; HUSSAIN, 2012). Mean levels reported for AFB_1 and OTA in rice in Vietnam was 3.31 μg/kg and 0.75 μg/kg, respectively (NGUYEN et al., 2007). The median level of DON in rice in the present study was below the tolerable limit for this mycotoxin in rice (750 μg/kg) in Brazil (ANVISA, 2011), and much lower than the median level reported for DON (116 μg/kg) by Almeida et al. (2012). In our work, a high percentage of positive samples for ZEN were found in rice (27%), with one sample containing 230 μg/kg, which is above the Brazilian MPL for this toxin (100 μg/kg).

However, the median level of ZEN in our study was similar to that reported by Almeida et al. (2012).

Concerning bean samples, low frequencies and levels were observed only for AFs and DON, and no quantifiable levels of the other mycotoxins evaluated were found in any bean sample. Low AF frequencies has also been reported in bean samples from the Brazilian state of Goiás (SILVA et al., 2002). However, a much higher frequency (75%) was reported in a previous study conducted in the Brazilian state of São Paulo (Jager et al., 2013), although the total AF mean level (0.10 $\pm$ 0.09 μg/kg) was lower than the value obtained in the present study (2.0 μg/kg). Results from studies conducted in other countries indicate higher occurrence of AFs in bean from Pakistan (LUTFULLAH; HUSSAIN, 2012), with 20% of red kidney bean and cowpea samples containing 5.0 and 2.2 μg/kg AFB_1, respectively.

Wheat flour had positive samples only for DON, OTA and FBs. The fact that five samples of wheat flour had levels above the Brazilian MPL (750 μg/kg) (ANVISA, 2011) warrants concern about the incidence of this toxin in wheat flour in Brazil. This result confirms the data reported by Santos et al. (2011), who observed a mean level of 1,385 μg/kg of DON in wheat flour from two states from Southern Brazil. Concerning corn flour, four types of mycotoxins were found in samples analyzed (AFs, DON, FB and ZEN), with one sample above the Brazilian MPL for total FB and two samples above the MPL for ZEN (1,500 and 150 μg/kg, respectively) (ANVISA, 2011). Corn meal also had positive samples for DON, FBs and ZEN, although no sample had levels above the respective MLPs for these toxins. These results indicate that corn products are important sources of exposure to multiple mycotoxins in small-scale farms in Brazil. FB levels above the Brazilian MPL in corn-based products in São Paulo were also observed by Bittencourt et al. (2005), who described mean concentrations of 6,200 ± 4,600 and 2,800 ± 2,000 μg/kg in corn meal and corn flour, respectively.

5.4.3 Estimates of mycotoxin exposure through food data

Risk assessment enables evaluation of the impact of the intake of food contaminated with mycotoxins on the health of the volunteers, leading to a different perspective on the occurrence of mycotoxin in foods. For total AF, the mean PDI values (Table 17) derived from rice and bean (0.013 ± 0.007 and 0.007 ± 0.007 μg/kg b.w./day, respectively) were higher than the PDI through the consumption of peanut products (0.00023 μg/kg b.w./day) in the Northeast region of São Paulo reported by Oliveira et al.

(2009). Exposure of general population to AF from all foods was reported in Europe ranging from 0.00093 to 0.00245 μg/kg b.w./day, in Africa from 0.0035 to 0.180 μg/kg b.w/day, in Asia from 0.0003 to 0.053 μg/kg b.w./day, and in the United States at 0.0027 μg/kg b.w./day (EFSA, 2007). These values are not too different in Brazil, as described with this study (ranging from 0.006 to 0.019 μg/kg b.w./day).

Regarding FBs, Bordin et al. (2015) analyzed samples of corn meal collected in two of the four cities of the present study (Pirassununga and Erval Velho), and reported lower PDI values for total FB (0.029 ± 0.037 μg/kg b.w./day) than the mean value obtained for this type of food in the present study (0.321 ± 0.312 μg/kg b.w./day). In another Brazilian southern state, PDI for total FB was similar to the values of the present study (0.121 μg/kg b.w./day) (MARTINS et al., 2012b). Considering the established TDI value for FBs of 2.0 μg/kg b.w./day determined by the FAO/WHO (2011a), consumption of food products evaluated in this study is considered safe for the population. However, the mean PDI for DON through wheat flour (1.002 ± 0.772 μg/kg b.w./day) was higher than the TDI for this mycotoxin (1.0 μg/kg b.w./day) (FAO/WHO, 2011b). The high occurrence of DON in wheat grains and flour in Brazil as described in previous studies (SANTOS et al., 2011; SANTOS et al., 2013; TRALAMAZZA et al., 2016) is in agreement with the results obtained in this work for samples of wheat flour.

5.4.4 Mycotoxin biomarkers in human urine

The analysis of urinary biomarkers revealed for the first time the presence of AFM_1, AFP_1, DON, DOM-1, OTA, FB_1 and ZEN in human urine in Brazil (Table 18). Risk assessment based on urinary mycotoxin levels have been performed in Brazil only for AF. Romero et al. (2010) confirmed the presence of AFM_1 in the urine of Brazilians (residents in a city of the state of São Paulo), with 78% of the analyzed samples presenting detectable levels of AFM_1. These results are higher than the present study which only showed 12%. In another study carried out in the state of São Paulo, Jager et al. (2014) also reported a higher frequency (61%) of samples with quantifiable levels (mean: 0.0012 ± 0.002 ng/mg creatinine). The percentage of positive samples reported in Northern Nigeria (14%) was more similar to the present study (EZEKIEL et al., 2014), although another report in this same country described a lower incidence (5%) and higher level (mean: 0.31 ng/mg creatinine) of urinary AFM_1 (WARTH et al., 2014).

5.4.5 Estimates of mycotoxin exposure through urinary levels

Urinary levels of AFM_1 indicated a lower PDI value (0.001 ± 0.002 μg/kg b.w./day) for AFB_1 (Table 19), when compared with the PDI estimated through food data (0.018 ± 0.001 μg/kg b.w./day). Jager et al. (2014) calculated the PDI for dietary AF in São Paulo state based on the AFM_1 levels in urine, obtaining values of 0.000034 and 0.000042 μg/kg b.w./day for men and women, respectively, which are ten times lower than that of the present study in São Paulo (mean PDI: 0.0004 ± 0.0003 μg/kg b.w./day).

As for DON and OTA, our results indicate that the mean PDIs based on urinary levels exceeded the established TDI values of 1.0 μg/kg b.w./day (FAO/WHO, 2011b) and 0.016 μg/kg b.w./day (FAO/WHO, 2007), respectively. Probable intakes greater than the TDI for DON based on urine samples has been reported in different parts of the world, such as Belgian (1.24 μg/kg b.w./day) (HEYNDRICKX et al., 2015), Italy (1.03 μg/kg b.w./day) (SOLFRIZZO; GAMBACORTA; VISCONTI, 2014) and South Africa (2.6 μg/kg b.w./day); (SHEPHARD et al., 2013). However, the PDI for DON presented in this study are much higher than the aforementioned estimates (84.914 μg/kg b.w./day). This fact could be due to the cultural differences in the intake of food products. According to data from the frequency questionnaire (Supplementary material A – ANNEX F), most of volunteers ate wheat-based bread every day. However, bread samples were not analyzed, and they may act as an additional source of exposure to DON. Heyndrickx et al. (2015) tested two formulas to calculate the DON PDI based on urine data and obtained different values: one below (0.62 μg/kg b.w./day) the TDI, and one above it (1.24 μg/kg b.w./day). Lower PDI estimates for DON were reported in Germany and the UK, with 0.52 μg/kg b.w./day (GERDING; CRAMER; HUMPF, 2014), and 0.729 μg/kg b.w./day (TURNER et al., 2008), respectively.

The higher probable intakes of DON, FBs and ZEN in the state of Santa Catarina shown in Figure 7 and 9 can be explained by some differences between daily food intakes and some climate differences. Food items that could possibly determine higher mycotoxin exposure in Santa Catarina according to the Food Consumption Frequency Questionnaires (Supplementary material A – ANNEX F) included boiled corn (32 g in SP and 71 g in SC), bread (58 g in SP and 103 g in SC) and cooked corn meal (35 g in SP and 81 g in SC). Pirassununga and Descalvado (SP) have lower annual temperatures (18-20^{o}C) and rainfall (1,300-1,600 mm), when compared with Pinhalzinho and Erval Velho (SC) (16-18^{o}C, 1600-1900 mm, respectively) (ALVARES et al., 2013), which could influence differently the fungi growth on food products in those areas.

5.4.6 Risk characterization

In our study, HQ values greater than 1 (indicative of health concern) in food samples was only observed for ZEN in the second sampling (Table 20), indicating tolerable levels of exposure for the other mycotoxins in both samplings. However, urinary biomarkers indicated that DON and OTA (first sampling) exceeded their tolerable levels of exposure since the HQ values were >1. Although FB and ZEN are frequently found in corn products in Brazil, their biomarkers in urine indicated a tolerable level of exposure of the individuals evaluated in the present study. Another important observation was the absence of OTA in food samples and its percentage exceeding the TDI in urine samples.

Uncertainties associated with exposure assessments need to be considered for the interpretation of results (DE BOEVRE et al., 2013). The major uncertainty in the present study should be associated with the difference between probable intake in food samples and the PDI in urine. These facts can be explained by possible additional daily exposure to mycotoxins by food sources not included in the sampling procedures, or by inhalation that may occur in several occupational settings of volunteers, such as handling animal feed (dairy cattle and poultry), or direct contact in grain production (BRERA et al., 2002; MAYER et al., 2008). However, two studies investigated the relationship of mycotoxin exposure between a control group with no occupational exposure, and a group of workers who were daily exposed at work. Both studies have concluded that mycotoxin levels and their biomarkers measured in urine mainly reflect dietary exposure (DEGEN, 2011; FÖLLMANN et al., 2016). Therefore, it remains to be determined if the inconsistences found between levels of DON, OTA and ZEN in food and PDI based on urine data on mycotoxin urine biomarkers are attributable to underestimation of consumption by the volunteers in the self-assessment questionnaire, rather than occupational exposure.

MoE of 0.019 for AFB_1 exposure in this study (Table 21) was quite different from values reported in other regions, such as Asian countries (833) (BENFORD; LEBBLANC; SETZER, 2010) and Malaysia (847) (LEONG et al., 2011). However, MoE values in the present study and those mentioned were below 10,000, which indicate a high risk of toxic outcomes as a consequence of the exposure to AFs (BENFORD; LEBLANC; SETZER, 2010). Although AFs assume a significant importance as carcinogenic food contaminants, there are few studies reporting population exposure to this mycotoxin (CANO-SANCHO et al., 2013). Previous studies identified peanuts as the main contributors to total intake of aflatoxins, worldwide (NJOBEH et al., 2010; OLIVEIRA et al., 2009; QIAN et al., 1994).

This is the first study describing the exposure to multiple mycotoxins and risk characterization in Brazil. The analysis of 203 food samples and 162 urine samples from adults living in rural areas revealed the presence of mycotoxins in 53% and 93% of the food and urine samples analyzed, respectively. Results demonstrated a clear exposure of this population to DON and high incidence of FBs in corn-based products. Although a low incidence of total AF was observed in food products, detectable concentrations indicated a potential health concern. However, uncertainties concerning the exposure assessment to AFs, ZEN and OTA highlight the need for future studies related to the exposure of the Brazilian population to multiple mycotoxins, in order to understand why occurrence and exposure levels in samples of food and urine samples showed inverted patterns for some mycotoxins, and to identify the main sources of exposure to mycotoxins in the Brazilian population. The present study may help Brazilian regulation agencies to better assess the mycotoxins in foods and exposure pattern in at least part of the population.

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6 Final Considerations

In the present study, multi-mycotoxin analytical methods were successfully validated and applied to evaluate the co-occurrence of mycotoxins in feed and food products available in rural areas of the states of São Paulo and Santa Catarina, as well as to assess the human exposure to mycotoxins through urinary biomarkers in those areas. High frequencies and relative low levels of mycotoxins were observed in food products, except for one sample of rice with ZEN, five wheat samples with DON and two samples of corn flour containing FB and ZEN at concentrations above their respective Brazilian MPL. Much higher frequencies and/or levels were observed in feed samples, hence emphasizing the need to improve the feed quality regarding the contamination with mycotoxins in small-scale farms in Brazil, especially for AF, FB and ZEN.

An important outcome of this study was the evidence of co-occurrence of mycotoxins in feed and food samples, which are presented in the Annexes H and I, respectively. Among the feed for broiler chicks, laying hens and dairy cattle evaluated, 98% of samples had at least one quantifiable mycotoxin, and 51% of samples had two or more mycotoxins (Annex H), which confirms a high degree of exposure of farm animals to mycotoxins in the feed in Brazil. For the food products analyzed, 38% of rice, 12% of beans, 97% of wheat flour, 100% of corn flour and 94% of corn meal presented at least one quantifiable mycotoxin. However, no mycotoxin co-occurrence was observed in bean samples (Annex I).

Detectable levels of mycotoxin biomarkers were found in 93% of urine samples evaluated, hence indicating a significant exposure of volunteers to dietary intake of mycotoxins, especially DON since it was quantified in 88% of samples analyzed. The co-occurrence of mycotoxin biomarkers was also observed in 61% of urine samples (Annex J), from which 8.2% contained four types of mycotoxins (AF + DON + OTA + FB; AF + DON + FB + ZEN; AF + DON + FB + DOM-1; DON + OTA + FB +ZEN) in the same sample.

Regarding the exposure assessment of humans to the evaluated mycotoxins, the PDI values based on food data were lower than those based on urinary levels of biomarkers. Therefore, the HQ obtained through the food samples indicated a non-tolerable exposure level only for 8 samples containing DON (10%), and 2 containing ZEN (4%). Considering the HQ calculated through the urinary levels, 49 samples with DON (15%) and 13 samples containing OTA (10%) would represent non-tolerable exposure levels for the mentioned mycotoxins. The differences between the PDI and HQ values for mycotoxins found in foods and respective biomarkers in urine suggest that the volunteers

in the present study were exposed to multiple mycotoxins through additional food sources other than those evaluated (rice, bean, wheat flour and corn products), or even occupational exposure in the farm environment. Another possible explanation for the differences found could be the low consistency or confidence in the volunteers' responses in the Questionnaire of Inquiry Reminder (IR24h - Annex C). Interestingly, we found differences in the PDI values between the volunteers from the states of São Paulo and Santa Catarina, which confirms the influence of food habits of the population on the actual exposure to dietary mycotoxins. However, when considering the results among sampling periods, we concluded that a simple, informal intervention by means of conversation and delivery of an information flyer (Annex E) was not enough to change the exposure levels to mycotoxins, possibly because of difficulties in changing and improving in-house food storage practices to avoid fungal development and mycotoxin production. In this case, it would require more in-depth intervention so that people incorporate the required knowledge on the mycotoxin contamination of foods.

In summary, the objectives proposed in the present work were accomplished, since the risk characterization based on food data analysis and urinary biomarkers indicated that human exposure to mycotoxins in the areas evaluated is a public health concern. Further studies are needed to identify food items other than those analyzed in this study as sources of dietary mycotoxins. Therefore, this thesis highlighted the importance of providing increasing knowledge on the risk assessment for mycotoxin mixtures in the diet of Brazilian population, also emphasizing the extension of multiple mycotoxin contamination of animal feed in small-scale producers as well as the urgent need for adopting regulations for feed in Brazil.

ANNEXES

ANNEX A – CEP/FZEA approval

FACULDADE DE ZOOTECNIA E ENGENHARIA DE ALIMENTOS DA USP

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Avaliação de Biomarcadores de Exposição Multi-Micotoxinas em Municípios dos Estados de São Paulo e Santa Catarina, Brasil

Pesquisador: Carlos Augusto Fernandes de Oliveira

Área Temática:

Versão: 1

CAAE: 54841616.3.0000.5422

Instituição Proponente: UNIVERSIDADE DE SAO PAULO

Patrocinador Principal: MINISTERIO DA CIENCIA, TECNOLOGIA E INOVACAO

DADOS DO PARECER

Número do Parecer: 1.500.317

Apresentação do Projeto:

O projeto trata de fazer uma análise pormenorizada das micotoxinas encontradas em alguns alimentos consumidos relacionando com a quantidade da mesma encontrada na urina de voluntários.

O trabalho é inovador, pois busca fazer a análise através de métodos analíticos utilizando LC-MS/MS que permite uma análise simultânea das diferentes amostras. Serão aplicados formulários sobre a frequência alimentar e hábitos alimentares e também será colhida informações sobre o consumo nas 24 horas que antecede a coleta de urina.

Objetivo da Pesquisa:

A pesquisa objetiva avaliar quais são as principais micotoxinas encontradas em alimentos e os resultados desta exposição na urina de voluntários. Essa análise será efetuada através de métodos analíticos usando LC-MS/MS.

Avaliação dos Riscos e Benefícios:

A pesquisa não oferece riscos ao voluntário, uma vez que estes não utilizarão nenhum alimento diferente do que é usualmente consumido por ele.

A pesquisa fornecerá aos voluntários folhetos ilustrativos visando reduzir a exposição às micotoxinas em alimentos, sendo este o principal beneficio direto a comunidade.

Endereço: Avenida Duque de Caxias Norte, 225
Bairro: Campus Universitário da USP **CEP:** 13.635-900
UF: SP **Município:** PIRASSUNUNGA
Telefone: (19)3565-4299 **Fax:** (19)3565-4114 **E-mail:** apoiofzea@usp.br

FACULDADE DE ZOOTECNIA E ENGENHARIA DE ALIMENTOS DA USP

Continuação do Parecer: 1.500.317

Comentários e Considerações sobre a Pesquisa:

A pesquisa é inovadora e os resultados são promissores, pois após a análise talvez seja possível demonstrar qual seria o padrão a ser empregados nos alimentos.

Considerações sobre os Termos de apresentação obrigatória:

Os documento obrigatórios foram apresentados corretamente e destacam os pontos principais da pesquisa.

Recomendações:

NDA

Conclusões ou Pendências e Lista de Inadequações:

O projeto está bem elaborado e plausível de ser executado.

Considerações Finais a critério do CEP:

O projeto atende os aspectos éticos na pesquisa com seres humanos portanto este Comitê recomenda sua aprovação. Caso haja alteração no número de indivíduos e/ou metodologia, favor relatar ao CONEP e ao CEPH.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_687601.pdf	01/04/2016 13:20:47		Aceito
Folha de Rosto	FolhaDeRostoAssinada.pdf	01/04/2016 13:20:26	Carlos Augusto Fernandes de Oliveira	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE.doc	29/03/2016 16:10:20	Carlos Augusto Fernandes de Oliveira	Aceito
Projeto Detalhado / Brochura Investigador	Projeto.doc	29/03/2016 16:07:46	Carlos Augusto Fernandes de Oliveira	Aceito

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

Endereço: Avenida Duque de Caxias Norte, 225
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PIRASSUNUNGA, 14 de Abril de 2016

Assinado por:
Daniele dos Santos Martins
(Coordenador)

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ANNEX B – Free and informed consent form

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

Você está sendo convidado(a) a participar, como voluntário(a), da pesquisa Avaliação de Biomarcadores de Exposição Multi-Micotoxinas em Municípios dos Estados de São Paulo e Santa Catarina, Brasil. No caso de você concordar em participar, favor assinar ao final do documento. Sua participação não é obrigatória, e a qualquer momento, você poderá desistir de participar e retirar seu consentimento. Sua recusa não trará nenhum prejuízo em sua relação com o pesquisador ou com a instituição.

Você receberá uma cópia deste termo onde consta o telefone e endereço do pesquisador principal, podendo tirar dúvidas do projeto e de sua participação.

Você têm direito a receber uma via do documento e que têm garantia de indenização diante de eventuais danos decorrentes da pesquisa, conforme artigos IV.3 e V.7 da resolução 466 de 2012 do CNS.

NOME DA PESQUISA: Avaliação de Biomarcadores de Exposição Multi-Micotoxinas em Municípios dos Estados de São Paulo e Santa Catarina, Brasil.

PESQUISADOR(A) RESPONSÁVEL: Prof. Dr. Carlos Augusto Fernandes de Oliveira

ENDEREÇO: Faculdade de Zootecnia e Engenharia de Alimentos- FZEA/USP. Av. Duque de Caxias Norte, 225 – Campus da USP, CEP 13635-900, Pirassununga/SP.

TELEFONE: (19) 3565-4173 / 4179

PESQUISADORES PARTICIPANTES: Larissa Tuanny Franco, Roice Eliana Rosim, Carlos Humberto Corassin, Tânia Petta, George Edwin Rottinghaus e Gilmar de Almeida Gomes

OBJETIVOS: O objetivo do trabalho é avaliar a interação das toxinas de fungos (micotoxinas) em condições de co-ocorrência nos alimentos com o organismo de seres humanos, conhecendo a dieta e investigando os resíduos e indicadores presentes na urina.

PROCEDIMENTOS: Se concordar em participar da pesquisa, inicialmente você terá que responder a um questionário sobre dados demográficos (gênero, idade, escolaridade), seus hábitos alimentares, qual a frequência e quantidade de produtos à base de grãos (exemplos: arroz, feijão, milho, trigo, amendoim e derivados), além de forma que adquire esses alimentos, plantio ou compra de produtos. Os procedimentos do estudo serão realizados da seguinte forma: será coletada uma amostra de aproximadamente 100 gramas de arroz, feijão, milho, trigo, amendoim e derivados (ou outros cereais, se disponíveis) antes de processar (cozinhar, cortar) e outra amostra desses alimentos prontos para o consumo, depois do processamento, no máximo 6 amostras por residência. No dia posterior, será coletada a amostra da primeira urina do dia. Será fornecido recipiente adequado para coletar a urina. O experimento será realizado 2 vezes durante 12 meses, isto é, depois de seis meses será realizada uma nova análise.

RISCOS E DESCONFORTOS: Existe um desconforto mínimo a você que se submeter à coleta de urina, sendo que se justifica pela necessidade de coletarmos esse material para sabermos o quanto da toxina permanece no nosso organismo e o quanto é excretado.

BENEFÍCIOS: Conhecendo o quanto o organismo absorve essa toxina temos suporte para estudar como podemos reduzir essa absorção podendo evitar possíveis doenças decorrentes dessa interação. Os resultados podem auxiliar também na elaboração de uma legislação para esse tipo de toxina, já que no Brasil ainda não existe nenhuma diretriz específica nessa área.

CUSTO/REEMBOLSO PARA O PARTICIPANTE: Você não terá que arcar com nenhum gasto decorrente da sua participação, além de fornecer uma amostra dos alimentos, nas 2

repetições do experimento. Você não receberá qualquer espécie de reembolso ou gratificação devido à participação na pesquisa.

CONFIDENCIALIDADE DA PESQUISA: Os pesquisadores irão tratar a sua identidade com padrões profissionais de sigilo. Seu nome ou o material que indique a sua participação não será liberado sem a sua permissão. Você não será identificado(a) em nenhuma publicação que possa resultar deste estudo. Serão divulgados dados diretamente relacionados aos objetivos da pesquisa.

Assinatura do Pesquisador Responsável: _____

CONSENTIMENTO DE PARTICIPAÇÃO DA PESSOA COMO SUJEITO

Eu, _____, RG_____ e CPF_____, declaro que li as informações contidas nesse documento, fui devidamente informado(a) pelo pesquisador(a) – Dr. Carlos Augusto Fernandes de Oliveira dos procedimentos que serão utilizados, riscos e desconfortos, benefícios, custo/reembolso dos participantes, confidencialidade da pesquisa, concordando ainda em participar da pesquisa. Foi-me garantido que posso retirar o consentimento a qualquer momento, sem que isso leve a qualquer penalidade. Declaro ainda que recebi uma cópia desse Termo de Consentimento.

LOCAL E DATA:

NOME E ASSINATURA DO SUJEITO OU RESPONSÁVEL (menor de 21 anos):

_____ _____

(Nome por extenso) (Assinatura)

ANNEX C- Questionnaire of Inquiry Reminder (RQ24h)

QUESTIONÁRIO DE INQUÉRITO RECORDATÓRIO DE 24 HORAS (IR24H)

Nome do Voluntário:	Data:

Quais os produtos abaixo, **que você forneceu amostras para o estudo**, você consumiu nas últimas 24 horas?

( ) Arroz (cozido). Quantidade:_____

( ) Feijão (cozido). Quantidade:_____

( ) Milho em grão (cozido, assado, etc). Quantidade:_____

( ) Fubá de milho. Quantidade:_____

( ) Bolo de milho. Quantidade:_____

( ) Outro alimento à base de milho (Especifique:_____). Quantidade:_____

( ) Bolo de farinha de trigo. Quantidade:_____

( ) Pão. Quantidade:_____

( ) Outro alimento à base de trigo (Especifique: _____). Quantidade:_____

( ) Amendoim em grão. Quantidade:_____

( ) Amendoim salgado (tipo japonês, frito e salgado, etc.). Quantidade:_____

( ) Amendoim confeitado (coberto com chocolate, colorido, etc.). Quantidade:_____

( ) Doce à base de amendoim (Especifique:_____). Quantidade:_____

( ) Paçoca. Quantidade:_____

( ) Outro alimento à base de amendoim (Especifique: _____). Quantidade:_____

( ) Outro alimento à base de cereais (Especifique: _____). Quantidade:_____

( ) Leite. Quantidade:_____

( ) Queijo (qualquer tipo). Quantidade:_____

( ) Outro alimento à base de leite (Especifique: _____). Quantidade:_____

ANNEX D - Food Consumption Frequency Questionnaires

QUESTIONÁRIO DE FREQUÊNCIA ALIMENTAR

1. Dados gerais

Nome:_____

Endereço:_____

Função:_____Telefone:_____

Data da Aplicação:_____

2. Quais os alimentos e as porções que você costuma comer? Por favor, responda a porção estimada.

Feijão cozido:

( ) ½ concha ( ) 1 concha ( ) 2 conchas Outros:_____

( )Nunca ( )Menos de 1 vez por mês ( )1 a 3 vezes por semana ( )2 a 4 vezes por semana ( )todos os dias ( )2 vezes ao dia

Arroz cozido:

( ) ½ xícara ( ) 1 xícara ( ) 2 xícaras Outros: _____

( )Nunca ( )Menos de 1 vez por mês ( )1 a 3 vezes por semana ( )2 a 4 vezes por semana ( )todos os dias ( )2 vezes ao dia

Polenta:

( ) 1 fatia ( ) 2 fatias ( ) 3 fatias Outros: _____

( )Nunca ( )Menos de 1 vez por mês ( )1 a 3 vezes por semana ( )2 a 4 vezes por semana ( )todos os dias ( )2 vezes ao dia

Massas (macarrão, caneloni, panqueca, etc.):

( ) ½ prato ( ) 1 prato ( ) 2 pratos Outros_____ ( )Nunca ( )Menos de 1 vez por mês ( )1 a 3 vezes por semana ( )2 a 4 vezes por semana ( )todos os dias ( )2 vezes ao dia

Pão francês, pão de forma, pão caseiro (preparados com farinha de trigo):

( ) 1 unidade /fatia ( ) 2 unidades./fatias ( ) 3 unidades./fatias Outros: _____

( )Nunca ( )Menos de 1 vez por mês ( )1 a 3 vezes por semana ( )2 a 4 vezes por semana ( )todos os dias ( )2 vezes ao dia

Bolo ou pão de milho:

( ) 1 fatia média ( ) 2 fatias médias ( ) 3 fatias médias Outros:_____

( )Nunca ( )Menos de 1 vez por mês ( )1 a 3 vezes por semana ( )2 a 4 vezes por semana ( )todos os dias ( )2 vezes ao dia

Creme de milho:

( ) ½ concha ( ) 1 concha ( ) 2 conchas Outros: _____

( )Nunca ( )Menos de 1 vez por mês ( )1 a 3 vezes por semana ( )2 a 4 vezes por semana ( )todos os dias ( )2 vezes ao dia

Farinha de milho amarela (cuscuz, cuscuz salgado, farofa):

( ) 2 colheres sopa ( ) 4 colheres sopa ( ) 6 colheres sopa Outros: _____

( )Nunca ( )Menos de 1 vez por mês ( )1 a 3 vezes por semana ( )2 a 4 vezes por semana ( )todos os dias ( )2 vezes ao dia

Milho enlatado:

( ) 1 colher sopa ( ) 2 colheres sopa ( ) 3 colheres sopa Outros:_____

( )Nunca ( )Menos de 1 vez por mês ( )1 a 3 vezes por semana ( )2 a 4 vezes por semana ( )todos os dias ( )2 vezes ao dia

Milho cozido:

( ) ½ espiga ( ) 1 espiga ( ) 2 espigas Outros:_____

( )Nunca ( )Menos de 1 vez por mês ( )1 a 3 vezes por semana ( )2 a 4 vezes por semana ( )todos os dias ( )2 vezes ao dia

Grãos (lentilha, grão de bico, soja e feijão branco):

( ) 1 colher sopa ( ) 2 colheres sopa ( ) 3 colheres sopa Outros:_____

( )Nunca ( )Menos de 1 vez por mês ( )1 a 3 vezes por semana ( )2 a 4 vezes por semana ( )todos os dias ( )2 vezes ao dia

Leite líquido:

( ) ½ xícara/copo ( ) 1 xícara/copo ( ) 2 xícaras/copos Outros:_____

( )Nunca ( )Menos de 1 vez por mês ( )1 a 3 vezes por semana ( )2 a 4 vezes por semana ( )todos os dias ( )2 vezes ao dia

Leite em pó:

( ) 1 colher sopa ( ) 2 colheres sopa ( ) 3 colheres sopa Outros:_____

( )Nunca ( )Menos de 1 vez por mês ( )1 a 3 vezes por semana ( )2 a 4 vezes por semana ( )todos os dias ( )2 vezes ao dia

Queijos:

( ) 1 fatia média ( ) 2 fatias médias ( ) 3 fatias médias Outros:_____

( )Nunca ( )Menos de 1 vez por mês ( )1 a 3 vezes por semana ( )2 a 4 vezes por semana ( )todos os dias ( )2 vezes ao dia

Iogurte:

( ) 1 pote ( ) 2 potes ( ) 3 potes Outros: _____

( )Nunca ( )Menos de 1 vez por mês ( )1 a 3 vezes por semana ( )2 a 4 vezes por semana ( )todos os dias ( )2 vezes ao dia

Flocos de milho (cereal matinal *sucrilhos* ou similares):

( ) ½ xícara/copo ( ) 1 xícara/copo ( ) 2 xícaras/copos Outros:_____

( )Nunca ( )Menos de 1 vez por mês ( )1 a 3 vezes por semana ( )2 a 4 vezes por semana ( )todos os dias ( )2 vezes ao dia

Barra cereal:

( ) 1 unidade ( ) 2 unidade ( ) 3 unidades Outros: _____

( )Nunca ( )Menos de 1 vez por mês ( )1 a 3 vezes por semana ( )2 a 4 vezes por semana ( )todos os dias ( )2 vezes ao dia

Bolo (preparado com farinha de trigo):

( ) 1 fatia média ( ) 2 fatias médias ( ) 3 fatias médias Outros: _____

( )Nunca ( )Menos de 1 vez por mês ( )1 a 3 vezes por semana ( )2 a 4 vezes por semana ( )todos os dias ( )2 vezes ao dia

Salgadinhos (*Fandangos*, *Cheetos* e similares, EXCETO BATATA FRITA):

( ) 1 pacote pequeno ( ) 1 pacote médio ( ) 1 pacote grande Outros:_____

( )Nunca ( )Menos de 1 vez por mês ( )1 a 3 vezes por semana ( )2 a 4 vezes por semana ( )todos os dias ( )2 vezes ao dia

Pipoca:

( ) 1 pcte pequeno ( ) 1 pcte médio ( ) 1 pcte grande Outros: _____

( )Nunca ( )Menos de 1 vez por mês ( )1 a 3 vezes por semana ( )2 a 4 vezes por semana ( )todos os dias ( )2 vezes ao dia

Canjica:

( ) ½ concha ( ) 1 concha ( ) 2 conchas Outros:_____

( )Nunca ( )Menos de 1 vez por mês ( )1 a 3 vezes por semana ( )2 a 4 vezes por semana ( )todos os dias ( )2 vezes ao dia

Amendoim (com casca, descascado, cru, tostado, pasta de amendoim):

( ) ½ xícara ( ) 1 xícara ( ) 2 xícaras Outros: _____

( )Nunca ( )Menos de 1 vez por mês ( )1 a 3 vezes por semana ( )2 a 4 vezes por semana ( )todos os dias ( )2 vezes ao dia

Paçoca:

( ) 1 unidade ( ) 2 unidades ( ) 3 unidades Outros_____ ( )Nunca ( )Menos de 1 vez por mês ( )1 a 3 vezes por semana ( )2 a 4 vezes por semana ( )todos os dias ( )2 vezes ao dia

Castanha-do-Pará, nozes, avelã e amêndoas:

( ) 1/4 xícara ( ) ½ xícara ( ) 1 xícara Outros: _____

( )Nunca ( )Menos de 1 vez por mês ( )1 a 3 vezes por semana ( )2 a 4 vezes por semana ( )todos os dias ( )2 vezes ao dia

Frutas desidratadas e secas:

( ) sim ( ) não

( )Nunca ( )Menos de 1 vez por mês ( )1 a 3 vezes por semana ( )2 a 4 vezes por semana ( )todos os dias ( )2 vezes ao dia

3. **Qual a origem dos alimentos de sua casa?**

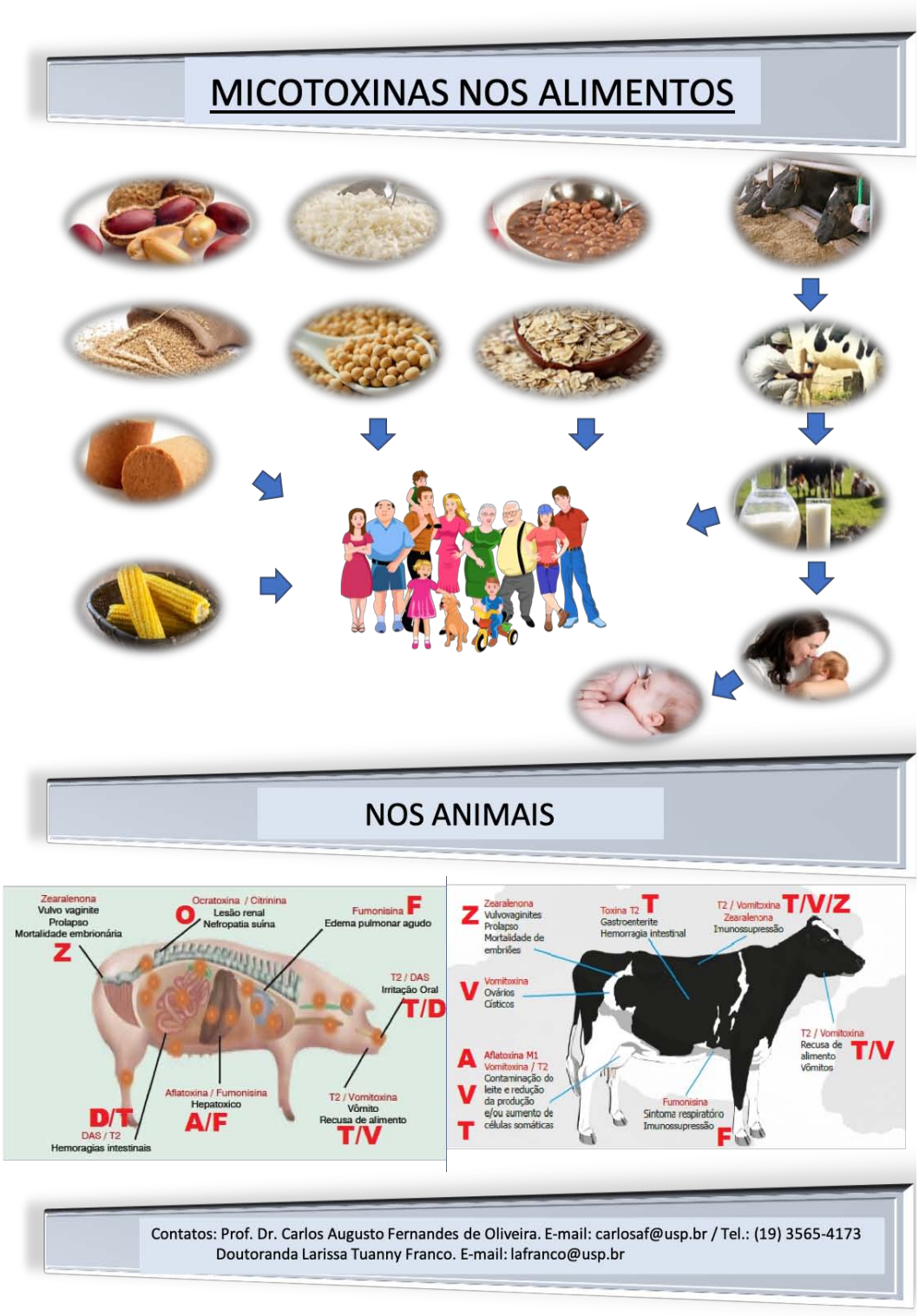
( )Supermercado	**( )Sacolão**	**( )Produção Própria**
( )Feira/ambulante	**( )Açougue**	**( )Armazém**
( )Produtor	**( )Padaria.**	Outros (espec.):

ANNEX E – FLYER

Front:

Source: Own authorship.

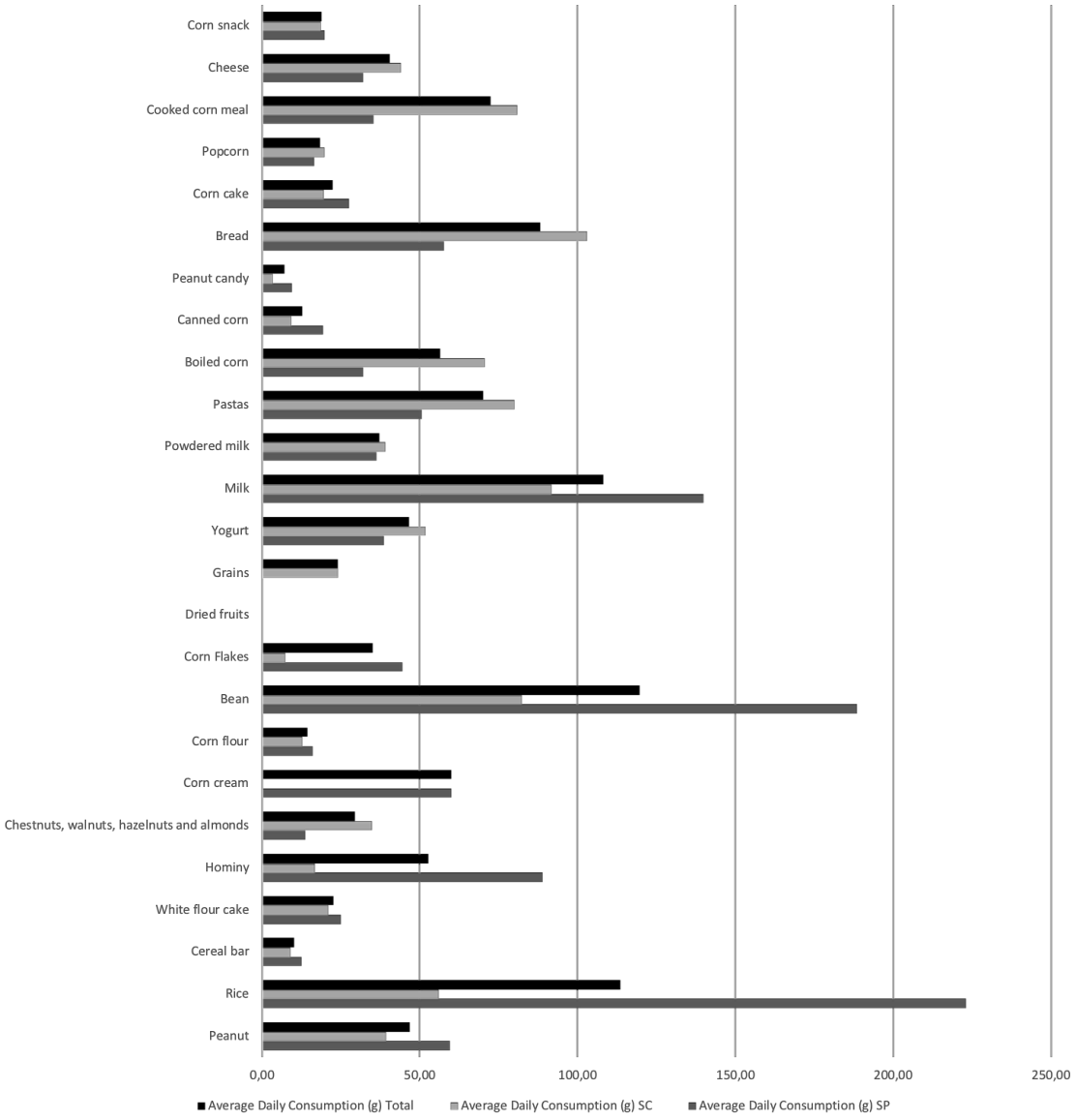
Verse:



Source: Own authorship.

ANNEX F - Dietary habits for the voluntaries according to the Food Consumption Frequency Questionnaires (Supplementary A)

Figure 10 - Dietary habits of volunteers in small-scale farms from the states of São Paulo and Santa Catarina, Brazil, based on the Food Consumption Frequency Questionnaires



Source: Own authorship.

ANNEX G – Food consumption data from each volunteer (Supplementary B)

Table 22 - Demographic and food consumption data from each volunteer in the first sampling period, based on the 24-h dietary recall questionnaire

continue

Person	Age	Weight (kg)	Genre	City	Rice (g)	Bean (g)	Corn flour(g)	Milk (mL)	Bread (g)	Cheese (g)	Wheat (g)	Penaut (g)	Corn meal (g)
ST1	49	80	F	Pirassununga	150	106	0	200	0	0	0	0	0
ST2	55	90	M	Pirassununga	150	106	0	200	0	0	0	0	0
ST3	54	114	M	Pirassununga	150	106	0	200	0	0	0	0	0
ST4	27	104	M	Pirassununga	150	106	0	200	0	0	0	0	0
ST5	21	103	M	Pirassununga	150	0	0	200	0	0	0	0	0
ST6	43	94	F	Pirassununga	150	106	0	200	0	0	0	0	0
ST7	46	83	M	Pirassununga	150	106	0	200	0	0	0	0	0
ST8	84	52	M	Pirassununga	150	106	150	0	0	0	0	0	0
ST9	78	52	F	Pirassununga	150	106	0	200	0	0	0	0	0
ST10	36	72	F	Pirassununga	150	106	0	200	50	0	0	0	0
ST11	62	98	M	Pirassununga	150	106	0	200	30	0	0	0	0
ST12	60	79	F	Pirassununga	150	53	0	200	30	0	0	0	0
ST13	73	56	M	Pirassununga	150	106	0	0	0	0	0	0	50
ST14	66	66	F	Pirassununga	150	53	0	100	0	0	0	0	50
ST15	37	51	F	Pirassununga	150	106	0	100	0	0	0	0	100
ST16	88	72	F	Pirassununga	150	53	0	100	0	0	0	0	0
ST17	41	75	M	Pirassununga	150	106	0	0	0	0	0	0	0
ST18	57	63	F	Pirassununga	300	106	150	100	0	0	0	0	0
ST19	28	83	M	Pirassununga	300	0	100	200	0	0	0	0	0

Table 22 - Demographic and food consumption data from each volunteer in the first sampling period, based on the 24-h dietary recall questionnaire.

continue

Person	Age	Weight (kg)	Genre	City	Rice (g)	Bean (g)	Corn flour(g)	Milk (mL)	Bread (g)	Cheese (g)	Wheat (g)	Penaut (g)	Corn meal (g)
ST20	19	48	M	Pirassununga	600	424	0	0	0	0	0	0	0
ST21	63	70	M	Pirassununga	150	106	0	0	0	0	0	0	0
ST22	58	60	F	Pirassununga	150	53	0	0	0	0	0	0	0
D1	33	62	F	Descalvado	75	106	0	0	0	0	0	0	0
D2	24	68	F	Descalvado	150	106	0	200	0	0	0	0	0
D3	35	93	F	Descalvado	150	0	0	400	0	0	0	0	0
D4	35	76	F	Descalvado	150	0	0	200	0	0	0	0	0
D5	37	87	F	Descalvado	75	0	0	200	0	0	0	0	0
D6	25	78	M	Descalvado	75	0	0	200	0	0	0	0	0
D7	27	68	F	Descalvado	75	212	0	200	0	0	0	0	0
D8	24	69	M	Descalvado	150	318	0	200	0	0	0	0	0
PI1	51	62	F	Pinhalzinho	225	106	0	0	60	60	0	0	0
PI2	51	92	M	Pinhalzinho	300	212	0	0	120	64	0	0	0
PI3	19	68	M	Pinhalzinho	0	424	0	0	30	32	0	0	0
PI4	23	57	F	Pinhalzinho	0	212	0	0	50	0	0	0	0
PI5	48	78	F	Pinhalzinho	150	212	0	0	0	64	200	0	0
PI6	51	68	M	Pinhalzinho	75	0	0	200	0	0	0	0	0
PI7	20	70	M	Pinhalzinho	75	212	0	200	0	0	0	0	0
PI8	59	80	F	Pinhalzinho	75	106	0	0	0	0	0	0	150
PI9	64	78	M	Pinhalzinho	0	0	0	0	60	64	0	0	50
PI10	49	69	M	Pinhalzinho	150	0	0	0	90	0	0	0	0
PI11	45	60	F	Pinhalzinho	150	0	0	0	60	64	0	0	0

Table 22 - Demographic and food consumption data from each volunteer in the first sampling period, based on the 24-h dietary recall questionnaire.

continue

Person	Age	Weight (kg)	Genre	City	Rice (g)	Bean (g)	Corn flour(g)	Milk (mL)	Bread (g)	Cheese (g)	Wheat (g)	Penaut (g)	Corn meal (g)
PI12	46	80	F	Pinhalzinho	75	53	0	0	30	64	0	0	0
PI13	22	50	F	Pinhalzinho	75	53	0	0	30	0	0	0	0
PI14	49	70	M	Pinhalzinho	150	53	0	100	0	64	0	0	0
PI16	58	75	M	Pinhalzinho	1500	53	0	0	30	0	0	0	0
PI17	57	58	F	Pinhalzinho	75	53	0	100	30	0	200	0	0
PI18	28	80	M	Pinhalzinho	150	53	0	100	60	0	0	0	0
PI19	33	68	F	Pinhalzinho	75	53	0	50	30	0	0	0	0
PI20	57	78	F	Pinhalzinho	75	0	0	200	50	0	0	0	0
PI21	31	57	F	Pinhalzinho	150	0	0	50	30	0	0	0	0
PI22	58	70	F	Pinhalzinho	75	106	0	200	30	0	0	0	0
PI23	67	73	M	Pinhalzinho	75	106	0	0	30	0	0	0	0
PI24	18	65	F	Pinhalzinho	75	106	0	0	30	0	0	0	0
PI25	54	55	M	Pinhalzinho	75	53	0	50	30	0	0	0	0
E1	27	87	M	Erval Velho	150	212	0	0	0	0	0	0	0
E2	21	51	F	Erval Velho	75	212	0	400	0	0	0	0	0
E3	75	50	F	Erval Velho	150	53	100	200	30	0	0	0	0
E4	78	90	M	Erval Velho	150	53	100	0	30	0	0	0	0
e32	42	63	F	Erval Velho	150	53	0	100	30	0	200	40	0
E7	45	110	M	Erval Velho	150	53	100	200	30	0	0	0	0
E5	31	50	F	Erval Velho	75	53	300	0	30	0	0	0	0
E6	26	100	M	Erval Velho	150	212	300	0	30	0	0	0	0
E8	44	70	M	Erval Velho	75	106	0	200	0	0	0	0	0
E9	45	72	M	Erval Velho	75	106	0	200	0	0	0	0	0

Table 22 - Demographic and food consumption data from each volunteer in the first sampling period, based on the 24-h dietary recall questionnaire.

conclusion

Person	Age	Weight (kg)	Genre	City	Rice (g)	Bean (g)	Corn flour(g)	Milk (mL)	Bread (g)	Cheese (g)	Wheat (g)	Penaut (g)	Corn meal (g)
E10	46	56	M	Erval Velho	75	106	0	200	0	0	0	0	0
E11	71	62	F	Erval Velho	75	106	0	200	0	0	0	0	0
E12	35	68	F	Erval Velho	75	106	0	200	0	0	0	0	0
E13	45	65	M	Erval Velho	75	106	0	0	0	0	0	0	0
E14	58	66	M	Erval Velho	0	53	100	0	0	0	100	0	0
E15	48	62	F	Erval Velho	0	53	100	0	0	0	100	0	0
E16	49	93	F	Erval Velho	150	53	150	200	0	0	0	0	0
E17	58	102	M	Erval Velho	150	106	150	200	0	0	0	0	0
E18	31	85	M	Erval Velho	150	106	0	0	50	0	0	0	0
E19	44	80	F	Erval Velho	150	106	0	0	50	0	0	0	0
E20	41	87	F	Erval Velho	150	106	0	0	50	0	100	0	0
E21	43	68	M	Erval Velho	150	106	0	0	50	0	100	0	0
E22	61	70	F	Erval Velho	75	53	100	200	100	0	0	0	0
E23	65	66	M	Erval Velho	75	106	100	200	150	0	0	0	0
E24	18	56	F	Erval Velho	75	53	0	200	0	0	0	0	0
E25	59	64	M	Erval Velho	75	212	0	300	50	0	0	0	0
E26	65	65	F	Erval Velho	300	212	0	100	0	0	0	0	0
E27	63	60	M	Erval Velho	300	212	0	100	0	0	0	0	0
E28	70	79	M	Erval Velho	75	106	0	100	0	0	0	0	0
E29	45	110	F	Erval Velho	150	106	0	0	0	0	0	0	0
E30	45	110	M	Erval Velho	150	106	0	100	100	0	0	0	0
E31	68	67	F	Erval Velho	150	106	0	100	100	0	0	0	0

Source: Own authorship. F: Female; M:Male.

Table 23 - Demographic and food consumption data from each volunteer in the second sampling period, based on the 24-h dietary recall questionnaire

continue

Person	Age	Weight (kg)	Genre	City	Rice (g)	Bean (g)	Corn flour(g)	Milk (mL)	Bread (g)	Cheese (g)	Wheat (g)	Penaut (g)	Corn meal (g)
ST1	49	80	F	Pirassununga	150	106	100	200	50	64	50	0	90
ST2	55	90	M	Pirassununga	150	212	0	0	0	32	0	0	0
ST4	27	104	M	Pirassununga	225	212	100	0	0	64	100	0	0
ST5	21	103	M	Pirassununga	59	0	0	280	60	64	42	0	0
ST6	43	94	F	Pirassununga	83	45	0	400	180	64	0	0	0
ST7	46	83	M	Pirassununga	0	0	0	200	50	0	42	10	0
ST8	84	52	M	Pirassununga	75	106	0	100	25	0	0	0	0
ST9	78	52	F	Pirassununga	75	106	0	200	25	0	0	0	0
ST10	36	72	F	Pirassununga	300	424	0	200	25	0	0	0	0
ST11	62	98	M	Pirassununga	0	0	0	200	0	0	0	0	0
ST12	60	79	F	Pirassununga	150	0	0	200	30	0	0	0	0
ST13	73	56	M	Pirassununga	150	106	0	200	0	32	100	0	0
ST14	66	66	F	Pirassununga	225	212	0	200	0	32	100	0	0
ST15	37	51	F	Pirassununga	150	212	0	50	0	32	100	15	0
ST17	41	75	M	Pirassununga	300	424	0	0	0	0	0	0	0
ST18	57	63	F	Pirassununga	225	318	0	500	150	50	0	0	0
ST19	28	83	M	Pirassununga	500	0	0	0	200	50	0	0	0
ST20	19	48	M	Pirassununga	300	424	0	600	150	0	0	0	0
ST21	63	70	M	Pirassununga	150	212	0	200	50	32	0	0	0
ST22	58	60	F	Pirassununga	150	106	0	0	50	32	0	0	0
D2	24	68	F	Descalvado	150	212	0	200	50	32	0	0	0

Table 23 - Demographic and food consumption data from each volunteer in the second sampling period, based on the 24-h dietary recall questionnaire.

continue

Person	Age	Weight (kg)	Genre	City	Rice (g)	Bean (g)	Corn flour(g)	Milk (mL)	Bread (g)	Cheese (g)	Wheat (g)	Penaut (g)	Corn meal (g)
D3	35	93	F	Descalvado	225	106	0	400	60	0	0	0	0
D5	37	87	F	Descalvado	59	53	0	200	50	0	0	0	0
D7	27	68	F	Descalvado	90	106	0	0	0	0	43	0	0
PI1	51	62	F	Pinhalzinho	225	0	0	0	60	32	80	0	0
PI2	51	92	M	Pinhalzinho	225	0	0	0	200	32	0	0	0
PI3	19	68	M	Pinhalzinho	400	0	0	0	50	0	0	0	0
PI5	48	78	F	Pinhalzinho	300	0	150	100	100	64	300	0	150
PI6	51	68	M	Pinhalzinho	150	212	0	200	60	32	100	0	250
PI7	20	70	M	Pinhalzinho	75	0	150	100	150	160	0	75	250
PI8	59	80	F	Pinhalzinho	150	106	0	0	30	0	0	0	0
PI9	64	78	M	Pinhalzinho	150	0	0	0	90	64	0	0	0
PI10	49	69	M	Pinhalzinho	225	0	0	0	200	0	100	0	0
PI11	45	60	F	Pinhalzinho	150	0	0	0	90	0	0	0	0
PI12	46	80	F	Pinhalzinho	300	0	0	0	50	32	0	0	0
PI13	22	50	F	Pinhalzinho	150	0	0	100	50	32	50	0	0
PI14	49	70	M	Pinhalzinho	300	0	0	0	50	0	0	0	100
PI16	58	75	M	Pinhalzinho	75	106	0	0	30	0	250	0	0
PI17	57	58	F	Pinhalzinho	75	106	0	0	60	0	150	0	0
PI18	28	80	M	Pinhalzinho	75	106	0	0	30	0	200	0	0
PI19	33	68	F	Pinhalzinho	75	0	0	0	30	0	100	0	0
PI21	31	57	F	Pinhalzinho	75	106	0	100	50	32	0	0	0

Table 23 - Demographic and food consumption data from each volunteer in the second sampling period, based on the 24-h dietary recall questionnaire.

continue

Person	Age	Weight (kg)	Genre	City	Rice (g)	Bean (g)	Corn flour(g)	Milk (mL)	Bread (g)	Cheese (g)	Wheat (g)	Penaut (g)	Corn meal (g)
PI22	58	70	F	Pinhalzinho	300	106	0	0	30	0	0	0	50
PI23	67	73	M	Pinhalzinho	150	45	0	0	15	0	0	0	50
PI24	18	65	F	Pinhalzinho	225	106	0	0	30	0	100	0	50
E1	27	90	M	Erval Velho	225	212	0	700	90	128	0	60	150
E2	21	51	F	Erval Velho	225	318	0	500	90	96	400	45	0
E3	75	60	F	Erval Velho	75	106	0	200	120	32	0	0	50
E4	78	85	M	Erval Velho	150	106	0	0	90	32	0	0	0
E32	42	63	F	Erval Velho	150	0	0	200	90	32	150	50	0
E7	45	110	M	Erval Velho	150	0	0	200	120	32	0	100	50
E5	31	40	F	Erval Velho	90	106	0	100	30	0	30	0	0
E6	26	110	M	Erval Velho	90	106	0	100	0	0	200	0	0
E8	44	71	M	Erval Velho	300	0	0	0	30	0	0	0	150
E9	45	74	M	Erval Velho	0	0	0	0	0	0	0	0	150
E10	46	55	M	Erval Velho	300	0	0	0	30	32	0	0	150
E11	71	64	F	Erval Velho	300	0	0	0	30	32	0	0	150
E12	35	73	F	Erval Velho	150	212	0	100	0	0	200	0	100
E13	45	68	M	Erval Velho	150	212	0	0	0	0	150	0	100
E14	58	62	M	Erval Velho	450	0	0	0	240	0	0	0	0
E15	48	67	F	Erval Velho	300	0	0	0	180	0	0	0	0
E16	49	93	F	Erval Velho	150	0	0	50	30	32	0	0	0
E17	58	102	M	Erval Velho	150	0	0	50	30	32	0	0	0

Table 23 - Demographic and food consumption data from each volunteer in the second sampling period, based on the 24-h dietary recall questionnaire.

conclusion

Person	Age	Weight (kg)	Genre	City	Rice (g)	Bean (g)	Corn flour(g)	Milk (mL)	Bread (g)	Cheese (g)	Wheat (g)	Penaut (g)	Corn meal (g)
E19	44	80	F	Erval Velho	0	0	0	0	60	0	0	0	50
E20	41	93	F	Erval Velho	300	0	0	0	30	0	0	0	0
E21	43	64	M	Erval Velho	300	0	0	0	30	0	0	0	0
E22	61	64	F	Erval Velho	150	0	0	50	30	32	200	0	100
E23	65	66	M	Erval Velho	0	0	0	100	60	32	0	0	100
E24	18	56	F	Erval Velho	150	0	0	600	0	0	90	50	0
E25	59	65	M	Erval Velho	300	424	0	50	150	0	0	0	100
E26	65	65	F	Erval Velho	300	424	0	100	30	32	42	0	150
E27	63	60	M	Erval Velho	300	424	0	100	30	32	42	0	150
E28	70	79	M	Erval Velho	300	0	0	100	30	32	0	0	100
E29	45	104	F	Erval Velho	500	0	0	0	90	0	0	0	0
E30	45	112	M	Erval Velho	300	0	0	100	30	32	0	0	0
E31	68	67	F	Erval Velho	75	0	0	25	30	0	0	0	100

Source: Own authorship. F: Female; M:Male.

ANNEX H – Co-occurrence of mycotoxins in feed samples

Table 24 - Number of mycotoxin co-occurrence in samples of broiler chicks, dairy cow and laying hens collected in small-scale farms from the states of São Paulo and Santa Catarina, Brazil.

Nº of Mycotoxin	Feed Product	Mycotoxin
2	Broiler chicks (n=1)	FB, DON
	Broiler chicks (n=1)	FB, ZEN
	Dairy cow (n=3)	FB, DON
	Laying hens (n=2)	FB, DON
	Laying hens (n=1)	FB, ZEN
	Laying hens (n=2)	AF, FB
	Laying hens (n=1)	AF, FB
3	Broiler chicks (n=1)	AF, FB, DON
	Broiler chicks (n=2)	FB, ZEN, DON
	Dairy cow (n=2)	FB, ZEN, DON
	Dairy cow (n=5)	FB, ZEN, DON
4	Dairy cow (n=1)	AF, FB, ZEN, DON
5	Dairy cow (n=1)	AF, OTA, FB, ZEN, DON

Source: Own authorship. AF: Aflatoxin. DON: Deoxynivalenol. OTA: Ochratoxin A. FB: Fumonisin. ZEN: Zearalenone.

ANNEX I – Co-occurrence of mycotoxins in food samples

Table 25 - Number of mycotoxin co-occurrence in samples of rice, beans, wheat flour, corn flour and corn meal collected in small-scale farms from the states of São Paulo and Santa Catarina, Brazil.

N º of Mycotoxin	Product	Mycotoxins
2	Rice (n=1)	AF, ZEN
	Rice (n=1)	FB, ZEN
	Rice (n=3)	DON, ZEN
	Wheat flour (n=3)	DON, OTA
	Wheat flour (n=5)	DON, FB
	Corn meal (n=1)	FB, ZEN
	Corn meal (n=2)	DON, FB
	Corn flour (n=1)	AF, FB
	Corn flour (n=5)	DON, FB
3	Corn flour (n=3)	DON, FB, ZEN
4	Corn flour (n=1)	AF, DON, FB, ZEN

Source: Own authorship. AF: Aflatoxin. DON: Deoxynivalenol. OTA: Ochratoxin A. FB: Fumonisin. ZEN: Zearalenone.

ANNEX J – Co-occurrence of mycotoxins in urine samples

Table 26 - Number of mycotoxin co-occurrence in samples of urine collected in small-scale farms from the states of São Paulo and Santa Catarina, Brazil.

N º of Mycotoxin	*n*	Mycotoxins
2	27	DON, OTA
	15	AF, DON
	10	DON, FB
	2	DON, ZEN
	1	OTA, FB
3	14	DON, OTA, FB
	8	AF, DON, FB
	7	AF, DON, OTA
	2	AF, DON, ZEN
	1	AF, DON, OTA
	1	AF, DON, Toxin T-2
	1	DON, FB, ZEN
	1	DON, OTA, ZEN
4	3	AF, DON, OTA, FB
	3	AF, DON, FB, ZEN
	1	AF, DON, FB, DOM
	1	DON, OTA, FB, ZEN

Source: Own authorship. AF: Aflatoxin. DON: Deoxynivalenol. OTA: Ochratoxin A. FB: Fumonisin. ZEN: Zearalenone.

ANNEX K - Biomonitoring of mycotoxin exposure using urinary biomarker approaches: a review. *Toxin Reviews*

Toxin Reviews

Biomonitoring of mycotoxin exposure using urinary biomarker approaches: a review

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Keywords:	Exposure assessment, Mycotoxins, Human biomonitoring, Urine, Biomarkers

ANNEX L - Performance and application of a "dilute-and- shoot" LC-MS/MS method for determination of mycotoxins in food products in São Paulo, Brazil. ***International Journal of Food Engineering***

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Performance and Application of a "Dilute-and-Shoot" LC-MS/MS Method for Determination of Mycotoxins in Food Products in São Paulo, Brazil

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***Abstract*—Mycotoxins are secondary metabolites produced by several species of fungi that occurs in agricultural commodities, which cause a great variety of toxic effects in vertebrates, including humans. In this study, a "dilute-and-shoot" method using ultra-performance liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) was evaluated and applied to assess the incidence of aflatoxins (AF) B_1, B_2, G_1 and G_2, fumonisins (FB) B_1 and B_2, ochratoxin A (OTA), zearalenone (ZEA) and deoxynivalenol (DON) in samples of corn meal ($N = 4$), corn flour ($N=2$), wheat flour ($N=4$), rice ($N=14$) and bean ($N=12$) collected in small-scale farms from São Paulo, Brazil. The performance parameters (aparent recovery, matrix effect, extraction recovery, linearity, limit of quantification and limit of detection) were satisfactory, and the most frequent mycotoxin detected in food samples was FB (7.11-316.04 μg/kg) below the Brazilian regulations. DON was detected in all samples of wheat flour (162.49-324.66 μg/kg) along with FB in 3 samples, and in one sample of bean (46.52 μg/kg). ZEA was detected in 2 samples of rice (4.90-6.78 μg/kg). Results indicated low incidences of mycotoxin in the products evaluated, although the co-occurrence of FB and DON warrants concern about their incidence in wheat flour in Brazil.**

***Index Terms*—aflatoxins, ochratoxin A, *Fusarium* toxins, occurrence, LC-MS/MS, analysis**

I. Introduction

Mycotoxins are secondary metabolites produced by fungi species that develop naturally in food products, which cause a great variety of toxic effects in vertebrates, including humans [1]. Exposure to toxins occurs predominantly by the ingestion of contaminated food, especially cereals and grains such as corn, wheat, and peanut, among others [2]. The most common toxigenic fungi found in Brazilian cereals include species from the genera *Aspergillus* and *Fusarium* [3]-[5].

(AFG_1) and G_2 (AFG_2), although AFB_1 has the higher acute, mutagenic and carcinogenic effects [3]. In Brazil, *A. ochraceus* (formerly *A. alutaceus*) is the main producing species of ochratoxin A (OTA), a toxin that interferes with the synthesis of macromolecules in the cells of the renal parenchyma, including DNA, RNA and proteins [1]. The *Fusarium* toxins are mycotoxins produced by several species in the genus, including the fumonisins, trichothecenes, and zearalenone (ZEA), among others [6]. Fumonisins are produced mainly by *Fusarium verticillioides*, being fumonisins B_1 (FB_1) and B_2 (FB_2) the most prevalent in grains under natural conditions, although FB_1 is the most toxic compound [7]. FB_1 has been associated with animal diseases such as equine leucoencephalomalacia and porcine pulmonary edema [2]. ZEA is an estrogenic substance derived from resorcylate acid, produced by several *Fusarium* species, e.g., *F. roseum* (*F. graminearum*), *F. culmorum* and *F. equisetum*, among others [7]. Deoxynivalenol (DON), also called vomitoxin, is a class B trichothecene mainly produced by *F. roseum*, which provokes nausea, diarrhea, reduced nutritional efficiency, gastrointestinal tract injuries and weight loss in animals [8].

Taking into account the risks posed by mycotoxins to the human health, several countries have determined tolerance limits for these toxins in food products. Brazilian regulations for mycotoxins were revised in 2011, with the introduction of maximum permitted levels (MPL) for aflatoxins (sum of AFB_1, AFB_2, AFG_1 and AFG_2), OTA, fumonisins (sum of FB_1 and FB_2), ZEA and DON in several food products [9]. Since the adoption of action levels for mycotoxins, a few reports in Brazil indicated high incidence rates (up to 100%) of fumonisins in corn products [4]. In another study [3], corn products showed 42% positive samples for aflatoxins at levels ranging from 0.05 to 8.3 μg/kg (sum of AFB_1, AFB_2, AFG_1 and AFG_2). Bean kernels had the highest incidence

ANNEX M – Co-occurrence of mycotoxins in maize food and maize-based feed from small-scale farms in Brazil: a pilot study. *Mycotoxin Research*

Mycotoxin Research
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ORIGINAL ARTICLE

Co-occurrence of mycotoxins in maize food and maize-based feed from small-scale farms in Brazil: a pilot study

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Abstract
A limited survey was conducted to assess the co-occurrence of aflatoxins (AF) B_1, B_2, G_1, and G_2; fumonisins (FB) B_1 and B_2; ochratoxin A (OTA); zearalenone (ZEN); and deoxynivalenol (DON) in maize food ($N = 26$) and animal feed ($N = 45$) collected from 21 small-scale farms from the states of São Paulo (SP) and Santa Catarina (SC), Brazil. Samples evaluated were maize meal and maize flour for human consumption available in the farm households, and maize-based feed intended for broiler chicks, laying hens, and dairy cows. Analyses of mycotoxins were performed by ultra-performance liquid chromatography coupled with tandem mass spectrometry. The median levels of mycotoxins found in maize food were 2.5 μg/kg (total AF), 120 μg/kg (total FB), 13 μg/kg (ZEN), and 57 μg/kg (DON). All values were below the Brazilian tolerance limits, except for total FB in one sample of maize flour. In feed samples, median levels of total AF, total FB, ZEN, and DON were 100 μg/kg, 680 μg/kg, 160 μg/kg, and 200 μg/kg, respectively. The co-occurrence of two or more mycotoxins was confirmed in 35% and 51% of maize food and feed, respectively. Results indicate a low human exposure to mycotoxins in the small-scale farms evaluated and a higher exposure of farm animals to mycotoxins in the feed.

Keywords Mycotoxins · Aflatoxins · Ochratoxin A · Fusariotoxins · Occurrence · Maize · Feed

Introduction

Mycotoxins are low molecular weight compounds with different chemical structures that are produced as secondary metabolites by several fungi species during growth on agricultural commodities, especially in cereal products (Abbas 2005). In Brazil, the most common toxigenic fungi found in cereals include species of the genera *Aspergillus* and *Fusarium*. *Aspergillus* species, mainly *A. flavus*, *A. parasiticus*, and *A. nomius*, produce aflatoxins (AF) B_1, B_2, G_1, and G_2, which have high toxicity, teratogenicity, mutagenicity, and carcinogenicity (Oliveira et al. 2014). *A. ochraceus* (formerly *A. alutaceus*) produces ochratoxin A (OTA), a toxin that interferes with the synthesis of macromolecules in the cells of the renal parenchyma (Pfohl-Leszkowicz and Manderville 2007). The mycotoxins produced by several species of the *Fusarium* genus, or fusariotoxins, include the fumonisins (FB) B_1 and B_2, deoxynivalenol (DON), and zearalenone (ZEN), among others, which exhibit a great variety of toxic effects in vertebrates including humans (Bryden 2012). Two or more mycotoxins may be present in cereal products because of multiple fungi contamination, or when one species produces more than one type of mycotoxin (Diaz 2005).

Maize and maize-based products are among the most vulnerable cereals to toxigenic fungi proliferation and subsequent contamination with single or multiple mycotoxins (Bryden 2012). In Brazil, previous studies indicated that the most frequently occurring mycotoxins in commercially available maize food are the aflatoxins (Jager et al. 2013; Souza et al. 2013) and fumonisins (Martins et al. 2012a; Bordin et al.

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ANNEX N - Assessment of Mycotoxin Exposure and Risk Characterization Using Occurrence Data in Foods and Urinary Biomarkers in Brazil. *Food and Chemical Toxicology*

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Abstract: This study aimed to assess the exposure of Brazilian residents (N = 86) from rural areas to multiple mycotoxins and to characterize the associated risk in two sampling periods (April-May and December/2016). Mean probable daily intake (PDI) values based on occurrence data in foods varied from 0.007 to 0.013, 0.069 to 1.002, 0.119 to 0.321 and 0.013 to 0.156 µg.kg-1 body weight (bw).day-1 for aflatoxins (AFs), deoxynivalenol (DON), fumonisins (FBs) and zearalenone (ZEN), respectively. Mean PDI values based on urinary biomarkers were 0.001, 84.914, 0.031, 0.377 and 0.002 µg.kg-1 bw.day-1 for AFB1, DON, ochratoxin A (OTA), FB1 and ZEN, respectively. Hazard quotient (HQ) based on occurrence data in food indicated a potential health concern (> 1) for ZEN in 2nd sampling. HQ values > 1 based on urinary biomarkers were observed for DON in the two samplings. Although OTA was not detected in any food sample, the HQ value based on urinary OTA levels was > 1 in 1st sampling. Margin of exposure values below 10,000 for AF resulted from food and urine data in the 1st sampling, indicating potential health risks. Future studies are needed to assess additional food and non-food sources of mycotoxin exposure in the population studied.