DOTTORATO DI RICERCA IN MALATTIE

INFETTIVE, MICROBIOLOGIA E

SANITÀ PUBBLICA

Biomonitoring data as a tool for assessing mycotoxins exposure of workers

Candidata

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A.A. 2017/2018

SUMMARY

3. MATERIALS AND METHODS

1. INTRODUCTION

Microscopic filamentous fungi, commonly known as molds, can develop on food commodities of plant origin (maize, wheat, etc.) and in some cases on commodities of animal origin (meat products, sausages). These molds can, in suitable environmental conditions, produce via secondary metabolism, chemical toxic compounds, known as mycotoxins. Mycotoxins can occur at pre-harvest, harvest and post-harvest stage.

Molecular structures of mycotoxins vary widely, so their effects on human and animal health also vary widely. Mycotoxins may be classified according to the target organ as hepatotoxins, nephrotoxins, neurotoxins, immunotoxins, or according to their toxicological effects as carcinogenic, genotoxic, mutagen, teratogen.

To date, more than 500 mycotoxins are known, even if researchers are focusing their scientific interest only to approximately 10 compounds, including aflatoxins and ochratoxin A due to their toxicity and widespread.

The most common route of exposure to mycotoxins is the ingestion through the diet by direct exposure, due to the consumption of contaminated food, or by indirect exposure through the consumption of food derived from animals fed with contaminated feedstuffs.

In addition to food diet, humans and animals can also be exposed to mycotoxins by inhalation of contaminated dusts. This phenomenon is particularly observed in certain working places such as harbors and warehouses. Several studies report a higher prevalence of lung carcinogenesis and bronchus and trachea tumors in

workers exposed to aflatoxins contaminated dusts. Exposure by inhalation and/or dermal contact is well known in different branches of industry, especially where a significant handling of dusty commodities such as grains, spices, coffee, etc., is performed.

Due to their severe toxicological implications, exposure to mycotoxins must be characterized by an accurate evaluation. Commonly, two different approaches can be followed for targeting this issue: via dietary exposure assessment and/or via biological monitoring. In both cases, a considerable number of uncertainties is present because of the lack of representativity in assessing the intake via food and the peculiar characteristics of toxicokinetics and toxicodynamics associated to the intake of the parent mycotoxins and the formation in vivo of the corresponding biomarker of exposure in the case of biomonitoring studies.

The present study aims to explore the validity of the biomonitoring studies as a tool to investigate the intake of mycotoxins in population groups such as workers operating in risky workplaces, being potentially exposed to mycotoxins through the inhalation of contaminated dust and/or by dermal contact. The objective is to produce accurate exposure data and perform exposure assessment of these population groups, by considering the fraction derived from the workplace environment by inhalation of dusts and/or by dermal contact.

In particular, this study was conducted on two groups of population, the exposed workers group that includes staffs working in an Italian feed plant, and a control group composed by administrative employees (non-exposed) working on the same feed plant.

Urine and serum samples were collected for the determination of mycotoxins, namely aflatoxin B_1, aflatoxin M_1 and AFB_1-N^7-Guanine adduct were analyzed in urine samples, while aflatoxin B_1, AFB_1-Lysine adduct and ochratoxin A were analyzed in serum samples.

The analytical determination of mycotoxins and their metabolites should be based on validated method with performance characteristics fitting for the purpose of biomonitoring. Therefore, the study also includes the set up and validation of suitable methods for the determination of the selected analytes in specimens (LC-HRMS). In particular, for urine analysis two methods were optimized and validated, a dilute&shoot method and an immunoaffinity clean-up method; for serum analysis a method based on liquid-liquid extraction and QuEChERS purification was developed and fully validated.

Moreover, due to the unavailability of commercial standard of AFB_1-N^7-Gaunine and AFB_1-Lysisne, the adducts were synthetized. Unfortunately, it was not possible to purify the synthesis products and to determine their concentrations; however, the obtained adducts were used for methods set up and for qualitative analysis (presence/absence) in the collected samples.

2. GENERAL ASPECTS

2.1. Mycotoxins

Microscopic filamentous fungi, commonly known as molds, can develop on food commodities of plant origin (maize, wheat, etc.) and in some cases also on commodities of animal origin (meat products, sausages). These molds can in suitable environmental conditions, produce chemical toxic compounds known as mycotoxins. The word mycotoxin stems from the Greek word μυκης, meaning mold, and τοξικόν meaning poison. Mycotoxins are naturally occurring secondary metabolites of some fungal species mainly belonging to the genera *Aspergillus*, *Penicillium* and *Fusarium*.

Essential factors for fungi proliferation and mycotoxin production are both the stress of the plant, as derived by the extreme soil dryness or the lack of a balanced nutrient absorption, and environmental factors such as climatic conditions (temperature, humidity and water activity) or mechanical damage of kernels as well as insects and pest attack (CAST, 2003). Mycotoxins can occur at pre-harvest, harvest and post-harvest stage.

Molecular structures of mycotoxins vary widely, so their effects on human and animal health also vary widely. Mycotoxins may be classified according to the target organ, as hepatotoxins, nephrotoxins, neurotoxins, immunotoxins, or according to their toxicological effects, as cancerogenic, genotoxic, mutagen, teratogen.

To date, more than 500 mycotoxins are known, even if researchers are focusing their scientific interest only to approximately 10 compounds, including aflatoxins and ochratoxin A due to their toxicity and widespread (Hajslova, 2011).

The presence of mycotoxins in a food commodity can occur even in the absence of a visible mold infestation due to a ceased vital cycle of the microorganism or by the effect of a removal of the mold due to technological processing of the food commodity. Nevertheless, the presence of a visible mold on the surface of a food product does not represent a clear indication of the presence of a mycotoxin. Generally, plant-origin commodities directly contaminated by mycotoxins are cereals, with maize as the riskiest crop being a staple food in many parts of the world, dried fruit, spices, grape, coffee, cocoa, fruit juices especially apple-based and, at minor extent, meat products and licorice. During the storage cycle, mycotoxins can directly contaminate also cheeses and sausages.

In addition, food products can become contaminated as a consequence of a carry-over from contaminated feeds and be present in food of animal origin such as milk, eggs and, at a minor extent, meat. It has to be noted that mycotoxins resist high temperatures and the common domestic cooking procedures are not able to destroy them.

2.1.1. Human exposure to mycotoxins

The most common route of exposure to mycotoxins is the ingestion through the diet by direct exposure due to the consumption of contaminated food, or by indirect exposure through the consumption of food derived from animals fed with contaminated feedstuffs.

In addition to food diet, humans and animals can also be exposed to mycotoxins by inhalation of contaminated dusts (Flannigan, 1996). This phenomenon is particularly observed in certain working places such as harbors and warehouses. Several studies report a higher prevalence of lung carcinogenesis and bronchus and trachea tumors in workers exposed to aflatoxins contaminated dusts (Lai, 2014; Liao, 2005; Van Vleet, 2001). Exposure by inhalation and/or dermal contact is well known in different branches of industry especially where a significant handling of dusty commodities such as grains, spices, coffee, etc., is performed, (Viegas, 2017; Viegas, 2015; Viegas, 2013; Viegas, 2012; Brera, 2002; Iavicoli, 2002). In this case, chronic bronchitis and asthma-like disorder and the accelerated decline of lung functions can occur together with other serious pathologies like cancer as well. However, there is a lack of epidemiological studies showing the relation between exposure and health effects, probably due to the fact that in the workplace, the risk assessment of mycotoxins has never been done routinely. In fact, while mycotoxin exposure via food intake is largely documented in the literature, the occupational exposure to these toxic compounds has been much less evaluated so far, despite its high frequency. Probably, this trend is due both to the fact that mycotoxins are not recognized as real and common occupational risk factor in specific settings (Viegas,

2018a) and to the difficulty to recruit volunteers prone in providing biological fluids.

Due to their severe toxicological implications, exposure to mycotoxins must be characterized by an accurate evaluation. Commonly, two different approaches can be followed for targeting this issue: via dietary exposure assessment and/or via biological monitoring. In both cases, a considerable number of uncertainties is present because of the lack of representativity in assessing the intake via food and the peculiar characteristics of toxicokinetics and toxicodynamics associated to the intake of the parent mycotoxins and the formation in vivo of the corresponding biomarker of exposure in the case of biomonitoring studies.

The dietary risk assessment is classically performed combining contamination data with consumption rates. The scenario that can be outlined, taking into consideration lower (LB) and upper (UB) boundaries of the contamination values, has multiple uncertainty sources such as the paucity of occurrence data available, censored dataset and their use, consumption rates not always updated and fit for purpose. Moreover, one other important source of uncertainty is associated to the variability coming from the sampling procedures carried out during the implementation of monitoring studies for data collection. This sampling drawback is of upmost importance since mycotoxins are characterized by an uneven distribution in the commodity lots, both in raw and in some processed commodities, too. In all cases, the uncertainty associated with the sampling step is much higher than the one associated with the analytical step.

Therefore, in consideration of the relevant problems associated with the sampling step, with the aim to get more direct information of the intake of single or multiple mycotoxins, the use of biomonitoring studies deserves a noteworthy mentioning.

While in the risk-based control programs, the classic deterministic method to estimate dietary exposure is accepted, in specific cases the biomonitoring studies, leading to the measurement of biomarkers of exposure, may represent a complementary method to directly confirm an exposure event or to substantiate the relevance or applicability of results derived from classical studies (Kroes, 2002). Moreover, researches on mycotoxins in biological fluids greatly contribute to clarify the mechanism of health impairment attributable to these toxic compounds and to elucidate the dose–response relationship (Miraglia, 1996).

The use of biomarkers is a tool that is still being explored in the mycotoxin field but is gaining more and more reliability for the most important mycotoxins in different population groups. Biomarkers represent a measure of the overall exposure and they are unable to discriminate between different sources of exposure (i.e. food or airborne) (Aitio, 1999) and in some cases this may represent a limit or a constrain to be taken into account. Biomarkers represent a parallel approach to the classic estimates of exposure based on food consumption and concentration levels, having the advantages of measuring exposure over time, estimating exposure directly (not relying on models and uncertainty assumptions) and assessing individual estimates (especially useful for specific subpopulations e.g. vegetarians, celiac patients).

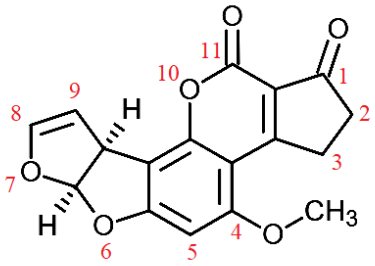
2.2. Aflatoxins

On the basis of their potent toxic effects aflatoxins, in particular aflatoxin B_1 (AFB_1) [CAS number: 1162-65-8], are historically the main toxins of concern since their characterization in 1958. These toxins are mainly produced by *Aspergillus flavus* and *Aspergillus parasiticus*, particularly in hot and humid areas, together with *Aspergillus nomius* and *Aspergillus pseudotamarii* (Ito, 2001; Kurtzman, 1987). Moisture higher than 85% and temperatures above 25°C are favorable conditions to the growth of aflatoxin producing fungi during storage (Diener, 1969). Aflatoxin B-hydroxilated metabolites, aflatoxins M_1 and M_2 [CAS number: 6885-57-0], are two toxins occurring in milk and derived products, as an effect of the fast metabolism of aflatoxins B_1 and B_2 (AFB_2).

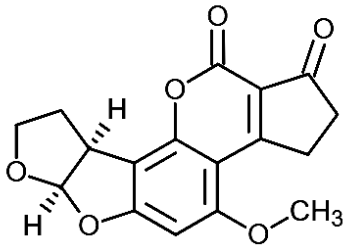
2.2.1. Physical and chemical properties

Aflatoxins (Figure 1) are substances that are chemically related to difuranocoumarin and classified in two broad groups according to their chemical structure; the difurocoumarocyclopentenone series, or B series, including AFB_1 and AFB_2 [CAS number: 7220-81-7], and the difurocoumarolactone series, or G series, including aflatoxin G_1 (AFG_1) [CAS number: 1165-39-5], aflatoxin G_2 (AFG_2) [CAS number: 7241-98-7] and aflatoxin M_1 (AFM_1) [CAS number: 6795-23-9]. The G series contains a D-lactone ring, while the B series contains a cyclopentenone ring, which is responsible for the major toxicity of the B series. The aflatoxins fluoresce strongly in UV light (ca. 365 nm), the B series produces a blue

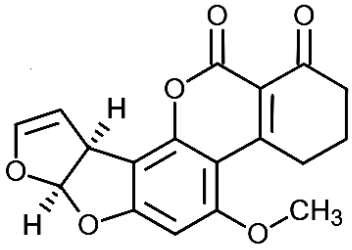
fluorescence whereas the G series produces green fluorescence. Aflatoxins are crystalline substances, insoluble in non-polar solvents, slightly soluble in water (10–20 µg/mL) and freely soluble in moderately polar organic solvents (e.g. chloroform, methanol), especially in dimethyl sulfoxide (Cole, 1981; O'Neil, 2001). Aflatoxins in dry state are very stable to heat up to their melting point. Pure aflatoxins are unstable to UV light in the presence of oxygen. Unstable to extremes of pH (< 3 or > 10). Unstable in the presence of oxidizing agents (Castegnaro, 1980, 1991). Under alkaline conditions, the lactone ring opens and the aflatoxins are apparently absent; however, the reaction is reversible upon acidification. Ammoniation, at high temperature and high pressure opens the lactone ring and results in decarboxylation, this reaction is not reversible.



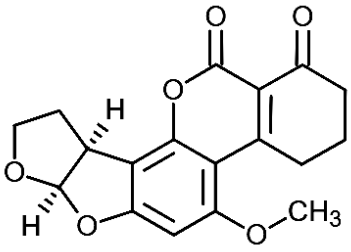
Aflatoxin B_1



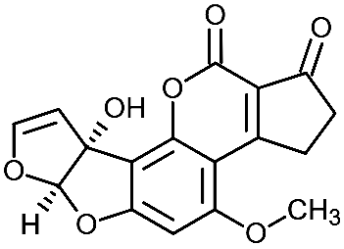
Aflatoxin B_2



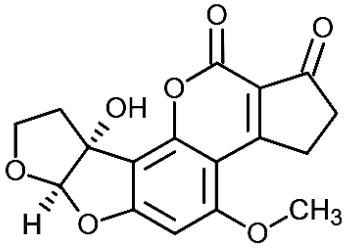
Aflatoxin G_1



Aflatoxin G_2



Aflatoxin M_1



Aflatoxin M_2

Figure 1. Chemical structure of aflatoxins.

2.2.2. Toxicity and metabolism

Aflatoxins are characterized by acute and chronic toxic effects. The target organ is the liver, AFB_1 is hepatotoxic, and several epidemiological studies related AFB_1 assumption to cellular hepatocarcinoma (HCC) that is the major cause of death in many parts of the world. AFB_1 is cancerogenic and genotoxic and is classified in group 1 from the International Agency for Research on Cancer (IARC, 1993).

Few cases of acute human poisoning were recorded, however there are known cases for animals, as Turkey X disease. Aflatoxicosis is characterized by hemorrhages, oedema, liver damage and can lead to death (IARC, 2012).

Aflatoxins metabolism has been extensively studied in animals and humans, this knowledge has provided the basis for development of biomarkers.

AFB_1 absorption occurs mainly in the small intestine (Wilson, 1985). Its permeability to the hepatocytes bring to accumulation in the liver, which is the principal organ for the xenobiotic transformation metabolism. Aflatoxins toxicity is activated by phase I of xenobiotic transformation metabolism. Among aflatoxins, AFB_1 is characterized by stronger toxic effects because of highly efficient metabolic activation with respect to the other forms.

AFB_1 is activated by cytochromes P450. The major CYP enzymes involved in human aflatoxin metabolism are CYP3A4 and 1A2. CYP3A4 is the predominant cytochrome in human liver and metabolizes AFB_1 mainly to exo-8,9-epoxide with much less efficient formation of AFQ_1 which represents the detoxification product (Wang, 1998). Also CYP1A2 can lead to formation of exo-epoxide, but also a

considerable amount of endo-epoxide is formed, as well as the hydroxylated AFM_1 (Gallagher, 1996; Ueng., 1995).

The exo-epoxide binds to DNA to form the predominant 8,9-dihydro-8-(N^7-guanyl)-9-hydroxy-AFB_1 (AFB_1–N^7-Guanine) adduct that confers the mutagenic properties to the compound. The positively charged imidazole ring of the AFB_1-N^7-Guanine adduct promotes depurination and, consequently DNA damage (Wang, 1999). The AFB_1-N^7-Guanine can be found in urine in a 3-day period and represents approximately the 0.2% of ingested AFB_1. AFB_1-N^7-Guanine is a validated biomarker of exposure for AFB_1 (Groopman, 1985).

AFM_1 is a poorer substrate for epoxidation and, consequently, is less mutagenic, carcinogenic and toxic than AFB_1. AFM_1 is the major AFB_1 metabolite excreted in milk and urine (1.2 and 2.2% of dietary AFB1, respectively) and is a validated biomarker of recent exposure (Groopman, 1985).

The exo- and endo-epoxides in blood are hydrolyzed with non-enzymatic reaction to AFB_1-8,9-dihydrodiol that form a dialdehyde phenolate ion.

Dialdehydes with a ring opening base-catalyzed reaction does not bind to DNA, but can form Schiff bases with primary amine groups for example with lysine, to form protein adducts such as aflatoxin–albumin (Wild, 2002). This adduct can be reduced by proteolysis at AFB_1-Lysine that is a biomarker of a 3-4-weeks period exposure; it was estimated that 1.4 – 2.3% of ingested AFB_1 is covalently bound to albumin (Wild, 1992).

Considering the metabolic pathway of aflatoxin B_1, it was decided to determine AFB_1, AFM_1 and AFB_1-N^7-Guanine adduct in urine and AFB_1 and AFB_1-Lysine adduct in serum samples.

2.3. Ochratoxin A

Ochratoxins A (OTA) [CAS number: 303-47-9], B and C (Figure 2) are compounds containing a phenylalanine moiety attached to a dihydroisocoumarin group via an amide bond. OTA, the toxin of most concern, also contains a chlorine atom on the aromatic ring, which accounts for its toxicity. Ochratoxins are produced by both *Aspergillus ochraceus* and *Penicillium viridicatum* (among others), with OTA being the most relevant toxin (Ciegler, 1973).

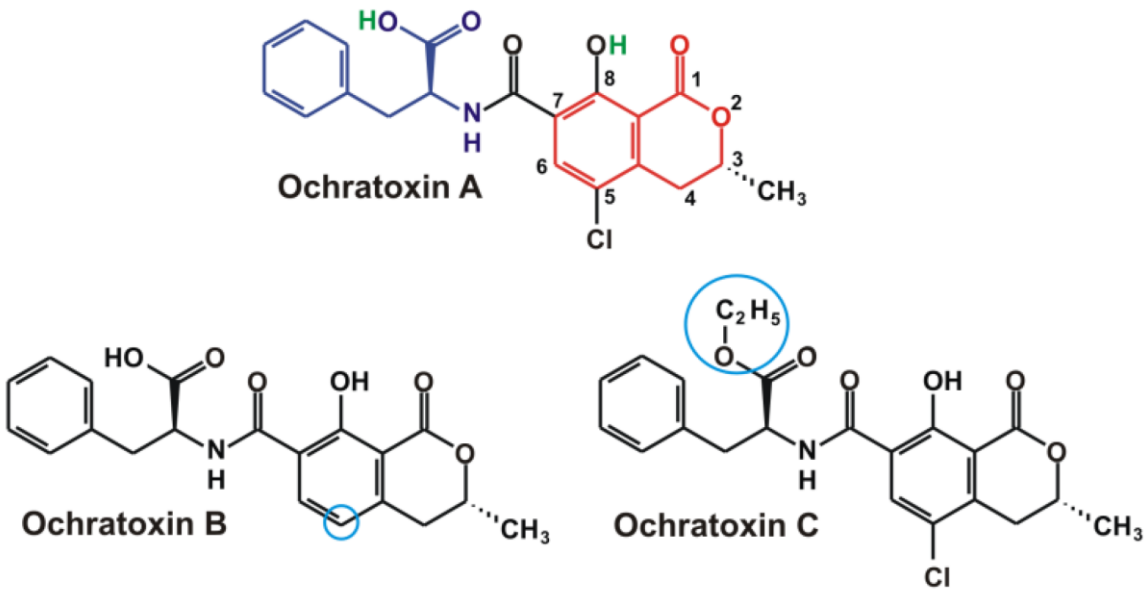


Figure 2. Chemical structure of ochratoxins.

2.3.1. Physical and chemical properties

OTA is a white odorless crystalline solid (Pohland, 1982), intensely fluorescent in UV light, emitting green and blue fluorescence in acid and alkaline solutions, respectively, due to two different forms, i.e. closed or open lactone ring, respectively. The melting point is 59°C when recrystallized from benzene–hexane (Natori, 1970); 169°C when recrystallized from xylene (Van der Merwe et al., 1965a, 1965b); 168–173°C after drying for 1 hour at 60°C (Pohland, 1982). OTA shows specific rotation with $[\alpha]^{20}_{D}$ –118° (c = 1.1 mmol/L in chloroform) (Van der Merwe, 1965a, 1965b); $[\alpha]^{21}_{D}$ –46.8° (c = 2.65 mmol/L in chloroform) (Pohland, 1982). As for the UV spectrum, at λ_{max} of 214, 282, and 332 nm, extinction coefficients of 37.2×10^{-3}, 0.89×10^{-3}, and 63.3×10^{-3} $L\times mol^{-1}\times cm^{-1}$, respectively, have been reported (Cole, 1981). OTA is moderately soluble in polar organic solvents (e.g. chloroform, ethanol, methanol) and is partially degraded under normal cooking conditions (Müller, 1983). The stability of OTA to heating conditions depends on the water activity of the medium (Subirade, 1996; Van der Stegen, 2001). The lactone ring opens under alkaline conditions, but the reaction is reversible. Solutions of OTA are completely degraded by treatment with an excess of sodium hypochlorite. Reaction in methanol and hydrochloric acid yields the OTA methyl ester, which can be used as a confirmatory reaction.

2.3.2. Toxicity and metabolism

The primary effect of OTA in all farm animals is nephrotoxicity. Fatty liver can occur in poultry. The most sensitive indicator of acute ochratoxicosis in chickens is the reduction in total serum proteins and albumin. A decrease in phosphoenolpyruvate carboxykinase in the kidney is a sensitive and specific indicator in pigs (Krogh, 1992; Marquardt, 1992).

The mechanism of action in farm animals is unclear. However, the structural similarity of OTA to phenylalanine and the fact that it inhibits many enzymes and processes that are dependent on phenylalanine, strongly suggest that OTA acts at least partially by disrupting phenylalanine metabolism (CAST, 2003; Riley, 2011).

In addition to inhibition of protein synthesis via binding to phenylalanine - tRNA synthetase, recent studies have demonstrated the ability of OTA to induce oxidative stress, reduce cellular defense, and alter signalling pathways involved in various aspects of cellular and mitotic regulation (Mally, 2009).

OTA is rapidly absorbed, the half-life in plasma depends on the extent of binding to plasma proteins. Published reviews have extensively summarized evidence on the absorption, distribution, metabolism, and mechanisms of action of OTA (IARC, 1993; Pfohl-Leszkowicz, 2007; Marin-Kuan, 2008; Mally, 2009).

Wide species differences have been reported in the serum half-life of OTA in vivo. In humans, the elimination of OTA follows a two-phase pattern, a fast excretion followed by a slow clearing, with a calculated plasma half-life of 35 days. Even infrequent exposure (consumption of contaminated food once a week or even once a month) can result in persistent blood levels of OTA (Studer-Rohr, 2000). Blood

samples from healthy people living in European countries show OTA levels in the range 0.1–40 ng/mL (WHO, 2008).

The parent molecule is the major compound found in blood, whereas ochratoxin α is the major component detected in urine (Studer-Rohr, 2000). OTA is absorbed from the gastrointestinal tract in mammals and becomes strongly bound to plasma proteins (predominantly albumin) in blood, whereby it is distributed to the kidneys, with lower concentrations in liver, muscle, and fat.

OTA is metabolized by several different CYP enzymes, depending on the species and tissue involved. In cells expressing human CYP enzymes, the main metabolite was 4(*R*)-hydroxy- OTA formed by CYP1A2, 2B6, 2C9, 2D6, and 2A6, whereas the 4(*S*)- hydroxy-OTA derivative was formed by only CYP2D6 and 2B6 (Pfohl-Leszkowicz, 2007).

Identified OTA metabolites include not only these two hydroxylated species but also 10-hydroxy-OTA and ochratoxin α, which is formed by hydrolysis of the peptide bond in OTA, the elimination of the phenylalanine moiety accounts for its non- toxicity.

The kidney is the major target organ for adverse acute effects of OTA (Pfohl-Leszkowicz, 2007; WHO, 2002). Short-term toxicity studies in mice, rats, dogs, and pigs have shown both time- and dose-dependent development of progressive nephropathy. Significant sex and species differences exist, as well as differences due to route of administration.

Other toxic effects include cardiac and hepatic lesions in rats, lesions of the gastrointestinal tract and lymphoid tissues in hamsters, myelotoxicity in mice, and

kidney lesions in chickens. Pigs appear to be the most sensitive species to the nephrotoxic effects; the lowest- observed-effect level (8 µg/kg bw) was used as the basis for establishing the Tolerable Weekly Intake (TWI) which was set by EFSA at 120 ng/kg bw (EFSA, 2006).

Due to the OTA strong interaction with serum albumin (yield>99%) (Chu, 1974), the presence of OTA itself in serum samples can be considered as a biomarker of exposure.

2.4. State of the art

Mycotoxins biomonitoring studies are mainly performed by LC-MS/MS, this approach allows to reach accuracy, selectivity and low LOD/LOQ values suitable for biological fluid analysis where mycotoxins content is quite low when compared to food and feed concentration levels (Capriotti, 2012; Warth, 2013). Recently, high resolution mass spectrometry (HRMS) methods for mycotoxins determination in biological fluids were also published.

In this study an Orbitrap Q-Exactive was used, this HRMS drives high resolution and accurate mass data, generating high resolution measurements of up to 500000 FWHM. When the instrument generates full-scan HRMS data during untargeted analysis, identification of novel compounds and retrospective data analysis are accomplished without the need to re-run samples. During targeted analysis scans, the instrument achieves confirmation and identification of residual small and large molecules (Righetti, 2016).

A research in the literature of methods on mycotoxin determination by LC-HRMS in biological fluids was performed before starting the methods set up for this project.

The first published method was the quantitative determination of zearalenone and its major metabolites in chicken and pig plasma samples (De Baere, 2012), followed by the study of Rubert et al. (2014) reporting a multi-mycotoxins method in milk also including AFB_1 and OTA determination. Slobodchikova and Vuckovic (2018) published a high resolution mass spectrometry method for monitoring 17 mycotoxins in human plasma including AFB_1, for which a LOD of 40 pg/mL_{serum}

was reported, and OTA, for which the obtained recovery factor was not acceptable and the toxin excluded from the quantitative determination. The recent work of McMillan et al. (2018) reports the determination of the AFB_1-Lysine adduct in serum samples of Nigerian children. None of the previous work is able to determine the needed mycotoxins/matrix combination, in addition the published methods report LOD/LOQ not fitting with the requirements set for this biomonitoring study. Regarding the sample preparation, for urine and serum purification and concentration different approaches are reported starting with light treatment and sample manipulation such as liquid-liquid extraction (LLE) (Meucci, 2005; Palli, 1999; Slobodchikova, 2018) that may be followed by further treatments such as QuEChERS (SLLE) (De Santis, 2017), or the employment of SPE cartidges (Brezina, 2014; Jager, 2016; McCoy, 2005). Also the use of mycotoxin specific immunoaffinity column (IAC), that allows to reach high selectivity and sensitivity but only on a very limited number of analytes, is reported (Ahn, 2010; Breitholtz, 1991; Dinis, 2007; Petkova-Bocharova, 2003; Sabbioni, 1990; Solfrizzo, 2011; Wild, 1992). Dilute&shoot approach may be conveniently applied for urine, providing that the LOD/LOQ requirements are fulfilled, but is not suitable for serum determination due to the matrix complexity (Gerding, 2015).

2.5. Aim of the work

The presented study was performed in the framework of the EFSA project "Biomonitoring data as a tool for assessing aflatoxin B1 exposure of workers – BIODAF" and aims to explore the validity of the biomonitoring studies as a tool to investigate the intake of mycotoxins in population groups, such as workers operating in risky workplaces, being potentially exposed to mycotoxins through the inhalation of contaminated dust and/or by dermal contact.

The objective is to produce a more accurate exposure and risk assessment of these population groups, by considering the fraction derived from the workplace environment by inhalation of dusts and/or dermal contact. The use of biomarker approach allows determining the possible contribution to the overall exposure due to potential professional exposure by enrolling for the study a group of professionally exposed workers as well as a control group composed of not exposed volunteers. The study also intends to contribute to obtain a more comprehensive and updated dataset on which management actions, aimed at minimizing the risk and improving the workplace conditions and workers' health, should be generated. In particular, this study was conducted on two groups of population, the exposed workers groups, that includes staffs working in an Italian feed plant, and a control group composed by administrative employees (non-exposed) working on the same plant. Urine and serum samples were collected for the determination of mycotoxins, AFB_1, AFM_1 and AFB_1-N^7-Guanine adduct were analyzed in urine samples, while AFB_1, AFB_1-Lysine adduct and OTA were analyzed in serum samples.

The analytical determination of mycotoxins and their metabolites should be based on validated method with performance characteristics fitting for the purpose of biomonitoring. Therefore, the study also includes the set up and validation of suitable methods for the determination of the selected analytes in specimens. In particular, for urine analysis two methods were optimized and validated, a dilute&shoot method and an immunoaffinity clean-up method; for serum analysis a method based on liquid-liquid extraction and QuEChERS purification was developed and fully validated.

Moreover, due to the unavailability of commercial standard of AFB_1-N^7-Gaunine and AFB_1-Lysisne, the adducts were synthetized. Unfortunately, it was not possible to purify the synthesis products and to determine their concentrations; however, the obtained adducts were used for methods set up and for qualitative analysis (presence/absence) in the collected samples.

The synthesis of AFB_1-N^7-Guanine and the validation of the dilute&shoot method for AFB_1, AFM_1 and AFB_1-N^7-Guanine analysis were included in the conclusive thesis of dr Gianmarco Mazzilli (AA 2016/2017), while the synthesis of AFB_1-Lysine adduct and the development and validation of the serum method were included in the conclusive thesis of dr Elisa Sonego (AA 2017/2018). As supervisor of dr Mazzilli and dr Sonego thesis I wish to thank them for the valuable work performed and the results obtained as well as for the contribution to this broader work.

3. MATERIALS AND METHODS

3.1. Samples

The investigation was conducted in a large feedstuff plant located in Northern Italy (Reggio Emilia), producing every year about 540000 metric tons of feedstuffs (e.g., flour, compost and pellet), nearly 100000 metric tons derived from maize.

The study was conducted under the supervision of the Local Health Unit of Reggio Emilia and was approved by the Ethical Committee of the Reggio Emilia Province. The plant management of the company agreed to participate according to the criteria and principles set by Italian legislation on workers' health and safety and the study on human samples was also agreed with trade union representatives and the competent medical team. The workers were informed about the purpose of the study through a public meeting. During the meeting, formal consent for participation was individually requested and signed.

Two groups of volunteers were selected, the exposed group corresponding to all workers in direct contact with some risky activities such as the downloading of the raw material, its handling and the cleaning procedures, and the control group corresponding to people working in the same company but, designated to perform other activities to be considered not risky for the absence of contaminated environmental dusts.

The collection of human urine and serum samples was carried out on the work site by a physician and a nurse. The urine of exposed workers was collected in the morning and delivered to the medical staff before starting the morning shift. At that time, a blood sample was taken. A total of 61 male volunteers were enrolled. Blood

and urine samples were collected on Monday and Friday morning of the same working week from 32 exposed workers (employees working in dusty plant areas) and 29 non-exposed workers (employees with administrative duties working far from the dusty areas) as control group. Monday was chosen since it reflects a situation characterized by a preceding two-days washing period and Friday was selected with the aim to verify the possible accumulation of mycotoxins intake over the week of sampling. Blood samples were collected in 10 mL cryogenic tubes and immediately transported in refrigerated boxes at 0°C to the analytical laboratory. Urine samples were collected using sterile tubes (VACUETTE® Urine System, 10 mL, Greiner Bio-One Gmbh, 4550 Kremsmüster, Austria). Serum and urine samples were stored at -20°C until analysis.

The mean value and range for age and body weight of the enrolled volunteers are reported in Table 1.

Table 1. Distribution of enrolled volunteers by group, age and body weight.

Volunteers	**Number of subject**	**Mean age (range); years**	**Mean body weight (range); kg**
Exposed	32	53 (32-65)	80.1 (62-99)
Non - exposed	29	48 (33-63)	83.4 (64-125)
Total	61	-	-

3.2. **Analytical method**

3.2.1. Apparatus

Beside the ordinary laboratory equipment, for the analytical determination a UHPLC-HRMS system was used. The analytes were separated with Ultra High Performance Liquid Chromatography (UHPLC) (Thermo Scientific™ Dionex Ultimate™ 3000; (©2016 Thermo Fisher Scientific Inc.). The UHPLC is interfaced with a High Resolution Mass Spectrometer (Orbitrap™ Q-Exactive™; ©2016 Thermo Fisher Scientific Inc).

The scheme of the Q-Exactive is reported in Figure 3.

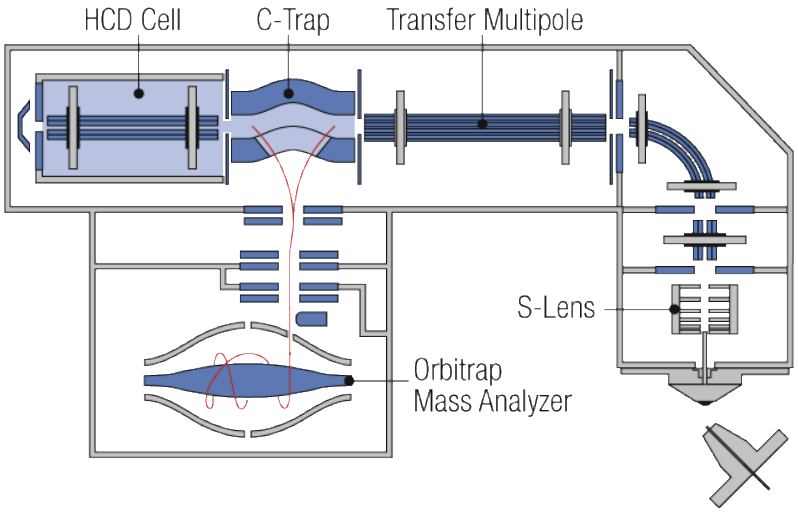


Figure 3. Schematic of the Q-Exactive mass spectrometer.

The Q-Exactive consists of 5 main components:

- Ion source (HESI II)
- Injection flatapole with mass resolving capabilities
- Quadrupole mass filter for precursor ion selection
- Intermediate storage device (C-Trap) for short pulse injection
- Collision cell for performing HCD (Higher Energy Collisional Dissociation) experiments
- Orbitrap analyzer for Fourier transform mass analysis

Sample is introduced into HESI II probe from the UHPLC system. The HESI II probe transforms ions in solution into ions in the gas phase by using electrospray ionization (ESI) in combination with heated auxiliary gas.

The ions are transferred into the C-Trap through four stages of differential pumping. The injection flatapole transmits ions from the source to the quadrupole, the injection flatapole also performs coarse pre-filtering of ions according to their m/z ratios. In the C-Trap, the ions are accumulated and their energy is dampened with a bath gas (N_2). The ions are then injected through three further stages of differential pumping by a lens system (Z-lens) into the Orbitrap analyzer where mass spectra are acquired by image current detection. The vacuum inside the Orbitrap analyzer is maintained below 1E-9 mbar. Ions are passed through the C-Trap into the HCD cell. The HCD cell adds a Higher Energy Collision Induced Dissociation capability to the instrument, this allows performing all-ion fragmentation (AIF) experiments.

After the ions have been fragmented in the HCD cell, the HCD cell voltages are ramped up and the ions are transferred back into the C-Trap from where they are injected into the Orbitrap analyzer for detection.

3.2.2. Chemicals and reagents

Chemicals and solvents used for sample preparation were 'pro-analysis' quality or better. LC-MS grade solvents, including water, methanol, acetonitrile (ACN) and formic acid (FA) were purchased from Fisher Scientific (Milano, Italy). The following reagents were purchased from Sigma-Aldrich (Darmstadt, Germany): ammonium formate, meta-chloroperoxybenzoic acid, protease from *Streptomyces griseus*, type XIV ≥3,5 units/mg, guanine (purity ≥98%), L-lysine (purity ≥98%). QuEChERS were from Waters (DisQuE, Waters, Milford, MA, USA).

The analytical reference standards of AFM_1 and OTA were purchased as stock solutions (0.5 and 10 µg/mL in ACN for AFB_1 and OTA respectively) from Biopure (Tulln, Austria). The internal standards U-[$^{13}C_{17}$]- AFB_1 (99.3% ^{13}C), U-[$^{13}C_{17}$]-AFM_1 (98.3% ^{13}C) and U-[$^{13}C_{20}$]-OTA (99.2% ^{13}C) were also purchased as ACN solution (0.5 µg/mL AFB_1 and AFM_1, 10 µg/mL OTA) (Biopure). Crystalline powder of AFB_1 from *Aspergillus flavus* (purity ≥98%) were purchased from Sigma-Aldrich (Darmstadt, Germany).

3.2.3. AFB_1-N^7-Guanine adduct synthesis

The AFB_1-N^7-Guanine adduct was synthetized since it was not commercially available at the moment of the study. The synthesis was made accordingly with Vidyasagar et al. (Vidyasagar, 1997) as follows: meta-chloroperoxybenzoic acid (MCPBA), 20 mg in 4 mL of dichloromethane, was washed with 100 mM phosphate buffer, pH 7.4 (4 mL x 4). The resulting MCPBA solution was passed through anhydrous sodium sulphate to remove residual water.

AFB_1 (0.64 µmoles) was dissolved in 250 µL of dichloromethane and was converted to AFB_1-8,9-epoxide (Figure 4) by addition of 250 µL of the above MCPBA solution (4 µmoles) and 500 µL of 100 mM phosphate buffer, pH 7.2. The reaction was carried out at 5°C for 100 min with continuous vigorous stirring. At the end of 100 min the buffer fraction was pipetted out.

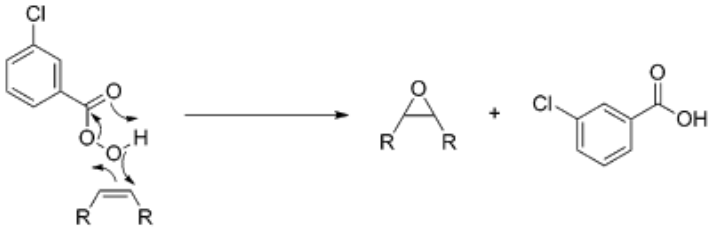


Figure 4. Electrophilic addition of oxygen to the double bond.

Guanine dissolved in 0.1 N HCl (0.32 µmoles) was taken in 500 µL of 100 mM phosphate buffer, pH 7.4 (maximum solubility of guanine in phosphate buffer was

found to be 140 µg/mL). The buffer with guanine was added to the tube containing AFB_1-8,9-epoxide in dichloromethane and the reaction was continued for 60 min at 5°C with continuous vigorous stirring. At the end of 60 min the reaction mixture was centrifuged at 4000 rpm for 5 min. The organic phase was separated and the buffer fraction was repeatedly washed with dichloromethane (500 µL x 3 times). Both the aqueous and organic phases were checked for the presence of AFB_1-N^7-Guanine adduct by UHPLC-HRMS (for experimental conditions see section 3.2.5.2). Chromatograms and spectra obtained for AFB_1-N^7-Guanine are reported in Figures 5 and 6, respectively. Due to the difficulties in assessing the concentration level of the synthetized adduct, it was used only for a qualitative evaluation of presence/absence in the collected urine samples.

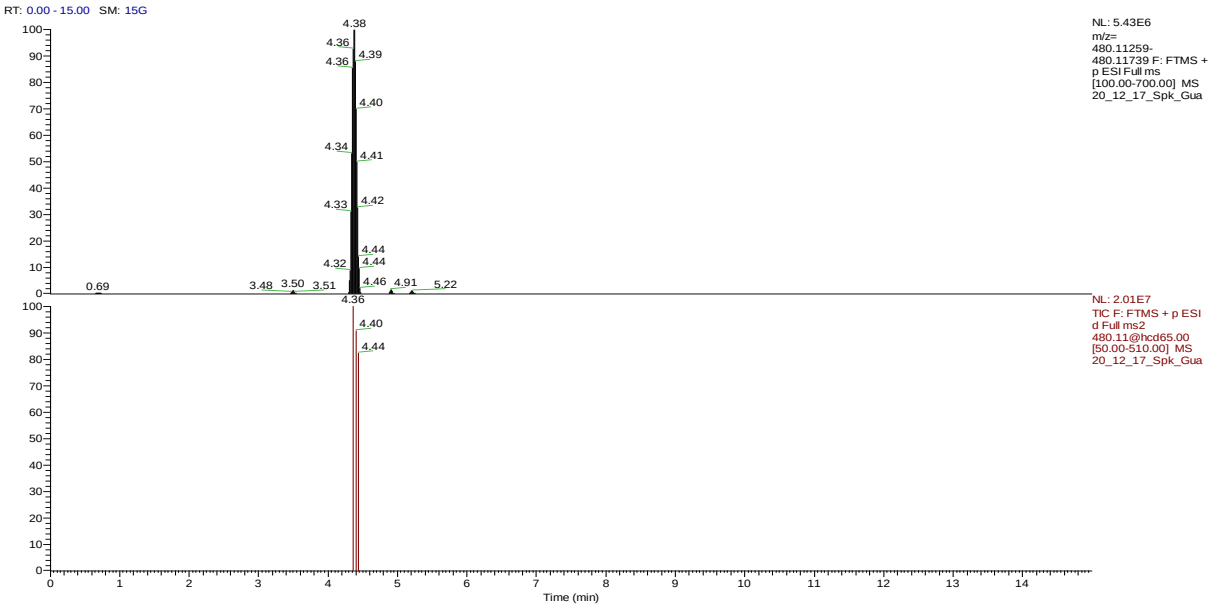


Figure 5. Extracted ion chromatogram for AFB_1-N^7-Guanine ($[M+H]^+$, m/z_{theo} = 480.11499, retention time 4.38 min).

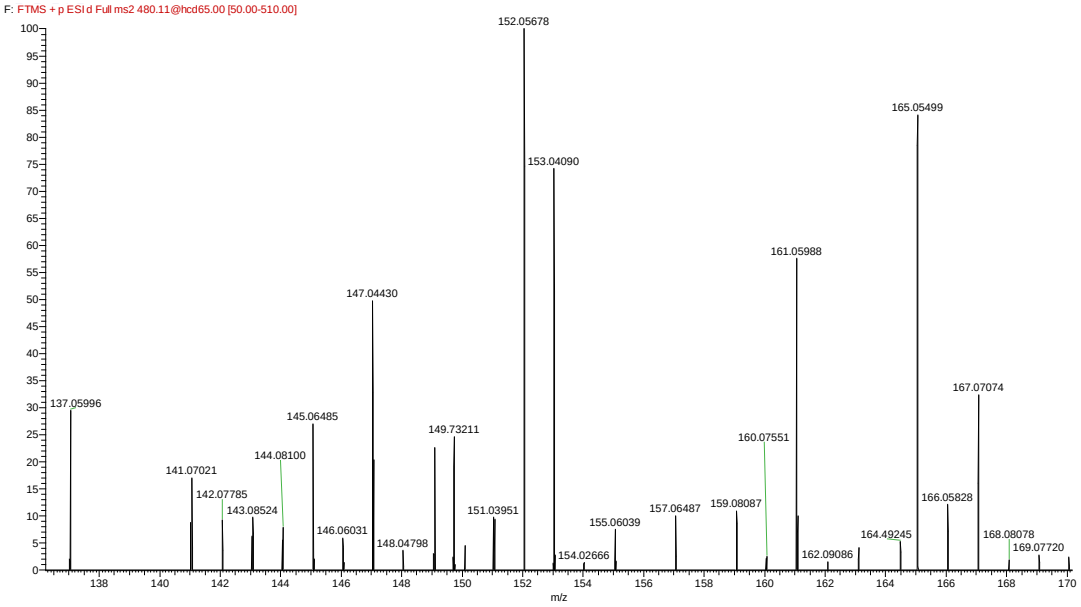


Figure 6. Data dependent mass spectrum (dd-MS^2) of AFB_1-N^7-Guanine.

3.2.4. AFB_1-Lysine adduct synthesis

The AFB_1-Lysine adduct was synthetized since it was not commercially available at the moment of the study. The synthesis was made accordingly with the procedures available in the literature and described by Sass et al. (Sass, 2014). The synthesis of the AFB_1-Lysine started by the preparation of AFB_1-8,9-epoxide as summarized in Figure 7.

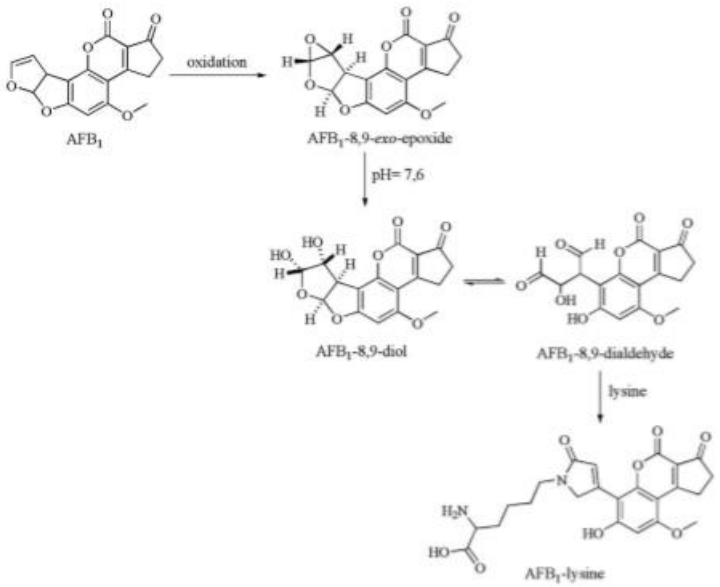


Figure 7. Steps for AFB_1-Lysine adduct synthesis.

The MCPBA (16 mg, 51 µmoles) was dissolved in 1 mL of dichloromethane and washed four times with phosphate buffered saline pH 7.4 (4 x 1 mL). The aqueous phase was removed and 0.8 mL of phosphate buffer (0.1 M, pH 7.6) was added to the organic phase.

The mixture was cooled to 0°C, and 2 mg of AFB_1 (6 µmoles) dissolved in 1 mL of dichloromethane was added. The reaction remained under agitation for 6 hours at 0°C. The aqueous phase was pipetted off, and the organic phase was dissolved in 1 mL of dichloromethane and washed with 0.5 M sodium thiosulfate (3 x 1 mL). Dichloromethane was removed by evaporation in a stream of nitrogen (Baertschi, 1988; Raney, 1992). Due to the instability of AFB_1-epoxide, the reaction mixture containing the AFB_1-8,9-exo/endo-epoxide was submitted to reaction with 0.8 mL of phosphate buffer (0.1 M, pH 7.6) to form the AFB_1-dialdehyde in equilibrium with AFB_1-diol, which are more stable than the corresponding AFB_1-epoxide (Scholl, 2008). After 20 min of stirring, the solution was washed with dichloromethane (3 x 0.8 mL) to remove the unreacted AFB_1. Afterwards, L-lysine (5 mg) previously dissolved in 0.4 mL phosphate buffer (0.1 M, pH 7.6) was added in the aqueous phase containing the products AFB_1-dialdehyde and AFB_1-diol (approximately 1 mg in total). The reaction remained under stirring for 24 hours to form the product AFB_1-Lysine. Chromatograms and spectra obtained for AFB_1-Lysine are reported in Figures 8 and 9, respectively. For experimental conditions, see section 3.2.6.2. Due to the difficulties in assessing the concentration level of the synthetized adduct, it was used only for a qualitative evaluation of presence/absence in the collected serum samples.

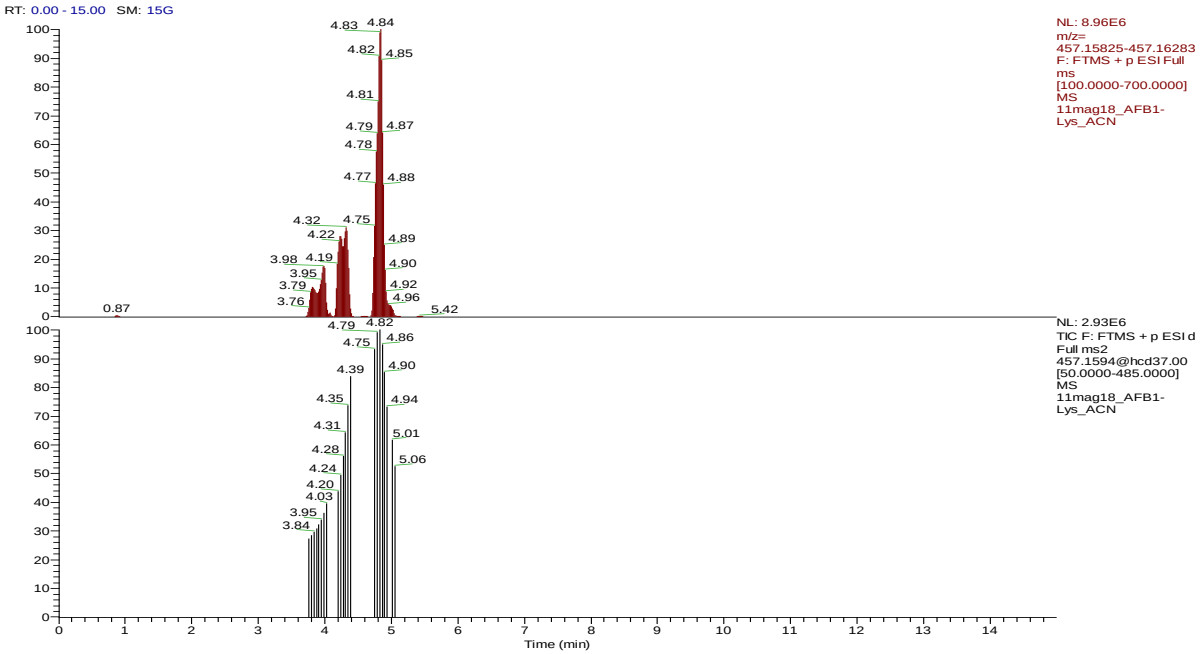


Figure 8. Extracted ion chromatogram for AFB_1-Lysine ($[M+H]^+$, m/z_{theo} = 457.16054, retention time 4.84 min).

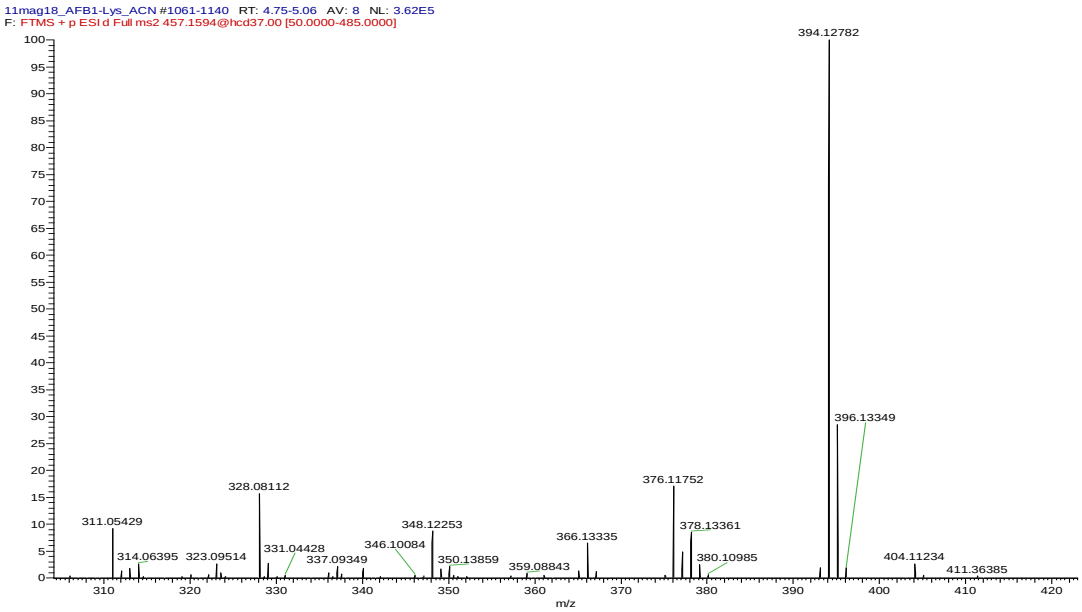


Figure 9. Data dependent mass spectrum (dd-MS^2) of AFB_1-Lysine.

3.2.5. Urine

3.2.5.1. Sample preparation

Dilute&Shoot

Urine samples were allowed to reach room temperature, a volume of 100 µL was mixed with 860 µL of H_2O LC-MS grade, for quantification purpose 20 µL of U-[$^{13}C_{17}$]-AFB_1 5 ng/mL in ACN and 20 µL of U-[$^{13}C_{17}$]-AFM_1 10 ng/mL in ACN were added to the sample. The diluted sample was centrifuged for 10 minutes at 10000 rpm before the injection of 10 µL into the UHPLC-HRMS system.

Immunoaffinity column clean-up

Urine samples were allowed to reach room temperature. Two mL of urine sample were mixed with 10 mL of phosphate buffered solution (PBS, pH=7.4) and applied to an IAC containing antibodies specific to AFB_1 and AFM_1 and tested for cross-reactivity with AFB_1-N^7-Guanine adduct (Easy-extract aflatoxins; R-Biopharm, Darmstadt, Germany). The IAC was washed with 30 mL of H_2O (10+10+10 mL), then the toxins were eluted with 1 mL of MeOH (500+500 µL). Five hundred µL of eluted sample were added with 20 µL U-[$^{13}C_{17}$]-AFB_1 2.5 ng/mL in ACN, 20 µL U-[$^{13}C_{17}$]-AFM_1 5 ng/mL in ACN and 460 µL of H_2O. Twenty µL of the sample were injected into the UHPLC-HRMS system. In Figure 10, the scheme of the sample preparation is reported.

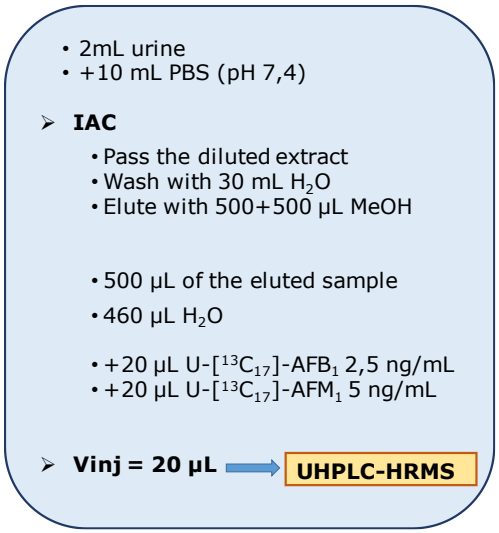


Figure 10. Scheme of the sample preparation for AFB1, AFM1 and AFB1-N7-Guanine analysis in urine with IAC clean-up method.

3.2.5.2. LC-HRMS analysis

Determination was performed by UHPLC-HRMS. Chromatographic separation was performed using UHPLC Dionex UltiMate 3000 (Thermo Scientific, San Jose, CA, USA) with Waters RP column Acquity BEH C18 (1.7 µm, 100 × 2.1 mm, Milford, MA, USA). The flow rate of 0.3 mL/min and the column temperature of 40° C were used for all analytes. The mobile phases A and B were water and methanol containing 0.002% formic acid and 2 mM ammonium formate. The following step gradient was used: 20% B increase to 99% in 10 min, keep isocratic at 99% B for 4 min, from 14 to 14.6 min return to 80% B, and finally re-equilibrate the column at 20% B for 2.4 min. The injection volume was set at 10 µL for dilute&shoot method and 20 µL for IAC method. High-resolution MS (HRMS) analysis was performed using Q-Exactive Orbitrap equipped with HESI source (Thermo Scientific, San Jose, CA, USA). The following ESI (+) parameters were

used: source voltage 3.5 kV, in-source CID 18 eV, NCE 50, capillary temperature 320°C, auxiliary gas heater temperature 350° C, sheath gas flow 40, S-lens RF level 75 and auxiliary gas flow 14. The MS acquisition was performed in Full Scan/Data Dependent (full MS/dd-MS^2) for confirmatory purpose. In this acquisition mode the Q-Exactive Orbitrap automatically switch between full scan (mass range 100-700 m/z; automatic gain control target 1×10^6 ions, and resolution of 70.000) and MS/MS acquisition, performing data-dependent scans. Precursor ions, selected by the quadrupole, are sent to the HCD collision cell; here they are fragmented to obtain ion spectra. At this stage resolution was set at 17.500 and automatic gain control target 2×10^5 ions. Normalized collision energy (NCE) was set at 25 and 27 for AFB_1 and AFM_1 respectively and at 40 and 90 for AFB_1-N^7-Guanine adduct. Precursor ion, fragments and collision energy (CE) used for the determination of the selected mycotoxins are reported in Table 2.

Table 2. Precursor ion, fragments and collision energy used for the determination of the selected mycotoxins in urine samples.

	Chemical formula	**Precursor ion (m/z) $[M+H]^+$**	**Fragment (m/z)**	**NCE[(a)]**
AFB_1	$C_{17}H_{12}O_6$	313.07066	285.07571; 241.04952	25
U-[$^{13}C_{17}$]-AFB_1	$C_{17}H_{12}O_6$	330.12770	-	-
AFM_1	$C_{17}H_{12}O_7$	329.06558 + 351.04752[(b)]	273.07538; 229.04937	27
U-[$^{13}C_{17}$]-AFM_1	$C_{17}H_{12}O_6$	346.12261 + 368.10456	-	-
AFB_1-N^7-Guanine	$C_{22}H_{17}N_5O_8$	480.11499	152.05678, 165.05499	40; 90

(a) NCE: Normalized Collision Energy
(b) $[M+Na]^+$

All analytical batches included analysis of appropriate extracts and solvent blanks, solvent calibration curves at the beginning and end of the analytical batch, and injection of a calibration level every 10 sample injections to ensure LC–MS stability throughout the run. For data acquisition and processing, Xcalibur software 4.0.27.19 was used. Mycotoxins were quantitated using the precursor ion which was extracted with ±5 ppm window. For AFM_1 the sum of the molecular ion and the $[AFM_1+Na]^+$ signals was considered.

The extracted ion chromatograms and dd-MS^2 TIC obtained by the injection of the highest calibration solution are reported in Figure 11. The data dependent spectra of AFB_1 and AFM_1 are reported in Figure 12 and 13, respectively. The extracted ion chromatograms of a urine sample naturally contaminated with AFM_1 is reported in Figure 14.

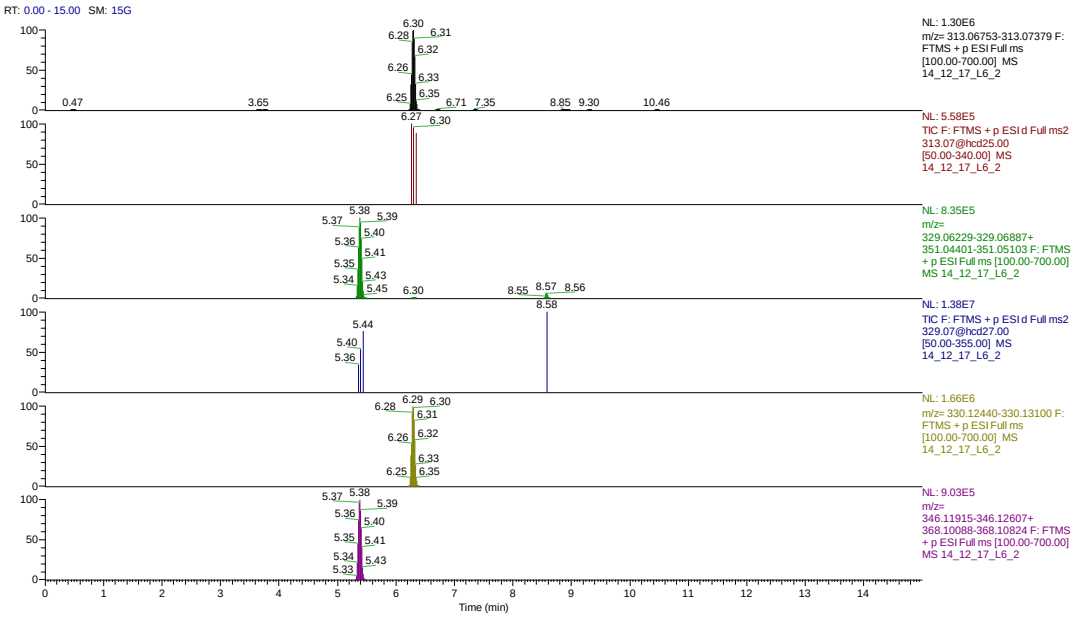


Figure 11. Extracted chromatograms obtained by the injection of the highest calibration solution. From the top AFB_1 peak followed by dd-MS^2 TIC of AFB_1 fragments, AFM_1 peak followed by dd-MS^2 TIC of AFM_1 fragments, the extracted ion chromatograms for U-[$^{13}C_{17}$]-AFB_1 and U-[$^{13}C_{17}$]-AFM_1, respectively.

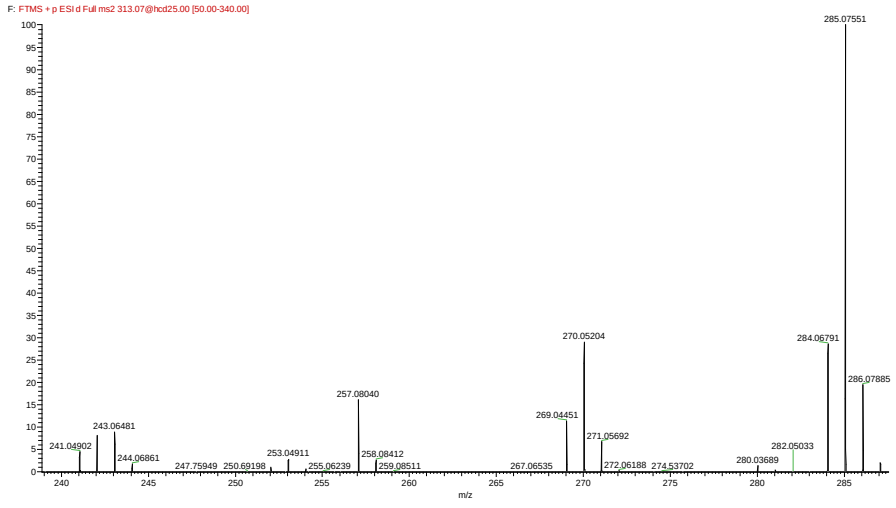


Figure 12. Data dependent mass spectrum (dd-MS^2) of AFB_1.

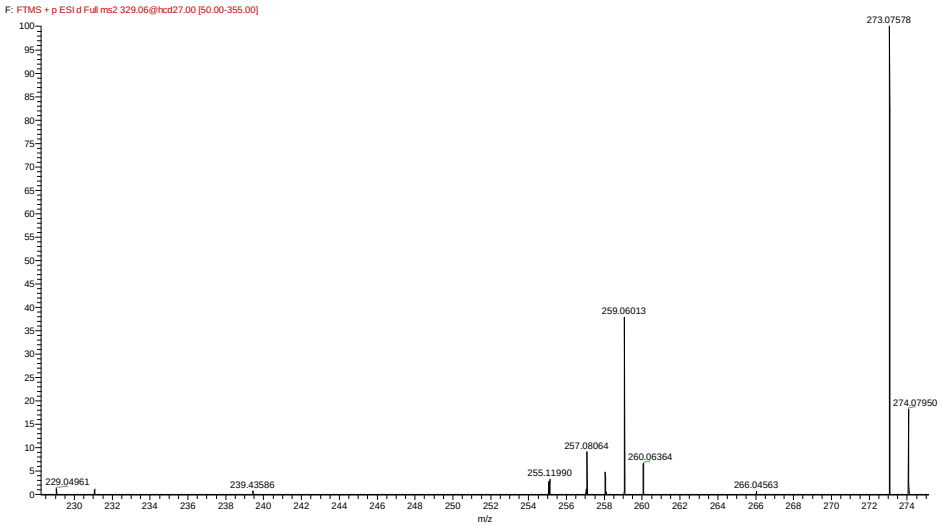


Figure 13. Data dependent mass spectrum (dd-MS^2) of AFM_1.

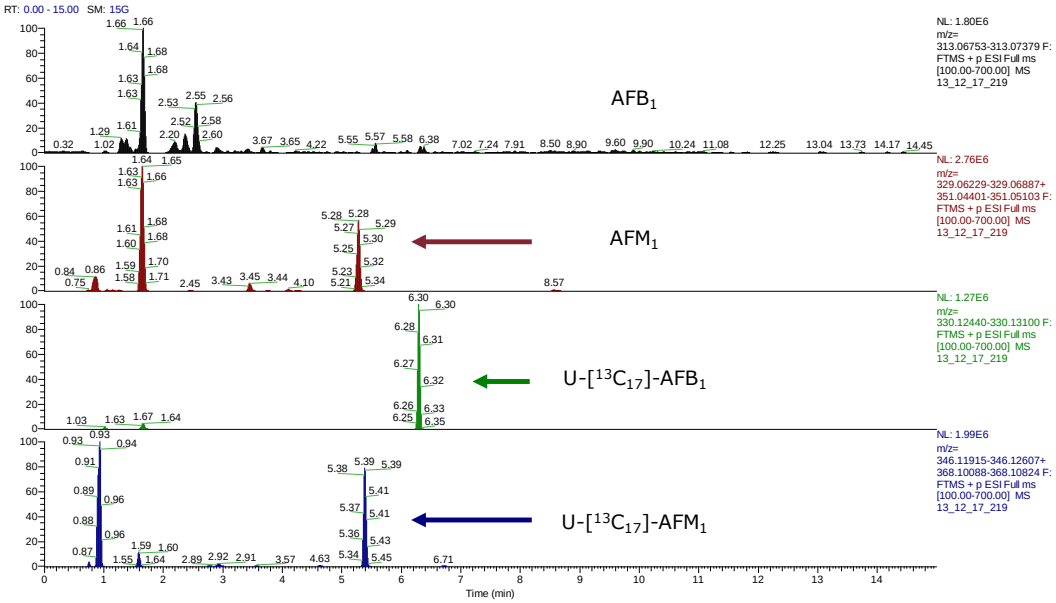


Figure 14. Extracted chromatograms obtained by the injection of a urine sample naturally contaminated with AFM_1. From the top the extracted ion chromatograms of AFB_1, AFM_1, U-[$^{13}C_{17}$]-AFB_1 and U-[$^{13}C_{17}$]-AFM_1, respectively.

3.2.5.3. Analytical quantification

For mycotoxins quantification an internal standard (ISTD) approach was adopted. The internal standard for AFB_1 and AFM_1 was the ^{13}C isotope labelled molecule in which all carbon atoms are substituted by the stable isotope ^{13}C. The calibration curve was obtained by plotting the ratio (standard area/^{13}C area) *versus* the concentration expressed in pg/mL_{urine}. The calibration curve was obtained by fitting the data with a linear regression model based on least squares method. ISTD was applied to the sample prior to extraction allowing to correct for extraction efficiency and matrix effects.

3.2.6. Serum

3.2.6.1. Sample preparation

Serum samples were allowed to reach room temperature. Five hundred µL were diluted with 200 µL of PBS (pH=7.4), mixed with pronase solution (100 µL, 40 mg/mL) and incubated in a water bath at 37°C for 18 hours. After enzymatic treatment with pronase, labelled internal standard solutions were added (20 µL U-[$^{13}C_{17}$]-AFB_1 5 ng/mL; 20 µL U-[$^{13}C_{20}$]-OTA 5 ng/mL). Serum sample was shacked for 5 min with 800 µL of n-hexane and centrifuged at 15000 rpm for 15 minutes at 4°C. The sample was then extracted in a 2 mL Eppendorf tube with 1 mL of acidified ethyl acetate (1% formic acid) by shaking for 30 min. The sample was centrifuged at 15000 rpm for 15 minutes at 4°C and the supernatant transferred in a collection amber vial. One mL of ACN was then added to the serum residue, sample is vortexed and mixed with 300 mg of QuEChERS (DisQuE, Waters, Milford, MA, USA). The sample was centrifuged at 15000 rpm for 15 minutes at 4°C and the organic layer transferred in a separate collection vial. Both collected organic phases were evaporated to dryness, reconstituted in 500 µL of MeOH:H_2O 10:90 v/v and 20 µL injected into UHPLC-HRMS system. In Figure 15, the scheme of the sample preparation is reported.

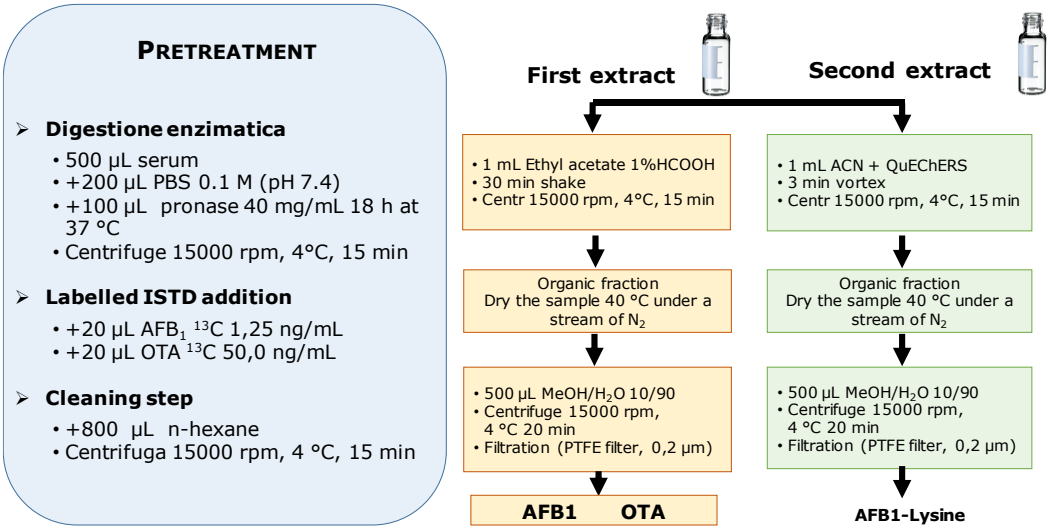


Figure 15. Scheme of the sample preparation for AFB_1, OTA and AFB_1-Lysine analysis in serum.

3.2.6.2. LC-HRMS analysis

Only slight modifications were applied for mycotoxins determination in serum with respect to the method previously reported for urine (Section 3.2.5.2). Namely, the following step gradient was used: 10% B increase to 99% in 10 min, keep isocratic at 99% B for 4 min, from 14 to 14.6 min return to 80% B, and finally re-equilibrate the column at 10% B for 2.4 min. The injection volume was set at 20 µL. For HRMS determination, the in-source fragmentation was not applied. Precursor ion, fragment and collision energy used are reported in Table 3.

The analytical method used for detecting AFB_1 in serum was also applied for the determination of OTA by the quantification of the free toxin. This extension to OTA was motivated by the explorative idea to evaluate a possible co-occurrence of AFB_1 and OTA in serum.

The extracted ion chromatograms and dd-MS^2 TIC obtained by the injection of the highest calibration solution are reported in Figure 16. The data dependent spectra of AFB_1 and OTA are reported in Figure 17 and 18, respectively. In Figure 19 the extracted ion chromatograms of a serum sample naturally contaminated with AFB_1 and OTA is reported.

Table 3. Precursor ion, fragments and collision energy used for the determination of the selected mycotoxins in serum samples.

	Chemical formula	**Precursor ion (m/z) $[M+H]^+$**	**Fragment (m/z)**	**NCE**
AFB_1	$C_{17}H_{12}O_6$	313.07066	285.07571; 241.04952	50
U-[$^{13}C_{17}$]-AFB_1	$C_{17}H_{12}O_6$	330.12770	-	-
AFB_1-Lysine	$C_{23}H_{24}N_2O_8$	457.16054	394.12782, 328.08112	37
OTA	$C_{20}H_{18}ClNO_6$	404.08954	257.02147; 239.01087	40
U-[$^{13}C_{20}$]-OTA	$C_{20}H_{18}ClNO_6$	424.15664	-	-

NCE: Normalized Collision Energy

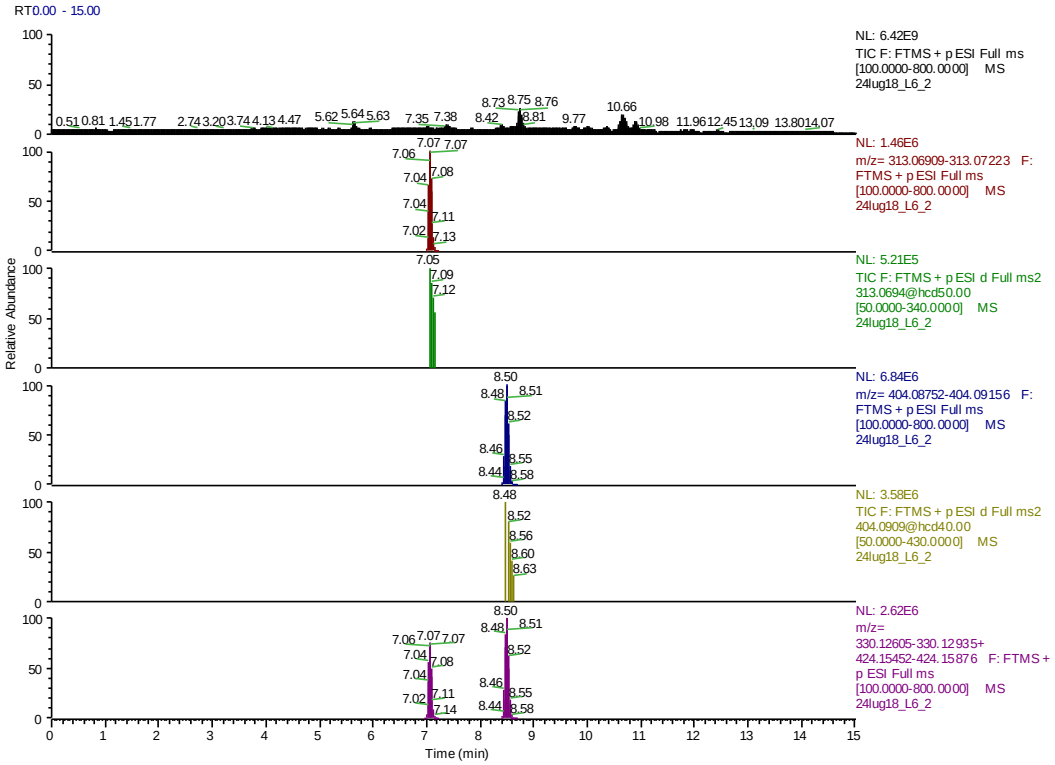


Figure 16. Extracted chromatograms obtained by the injection of the highest calibration solution. From the top TIC of the full scan in the range 100-800, AFB_1 peak followed by dd-MS^2 TIC of AFB_1 fragments, OTA peak followed by dd-MS^2 TIC of OTA fragments, the extracted ion chromatogram for U-[$^{13}C_{17}$]-AFB_1 and U-[$^{13}C_{20}$]-OTA.

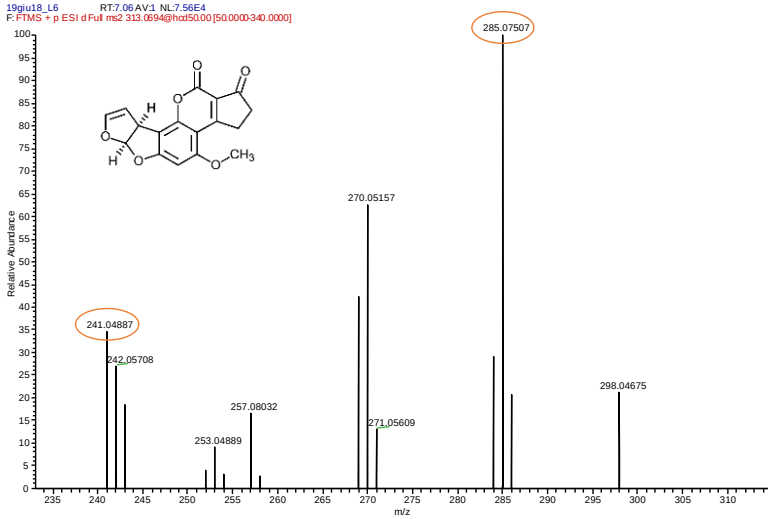


Figure 17. Data dependent mass spectrum (dd-MS^2) of AFB_1.

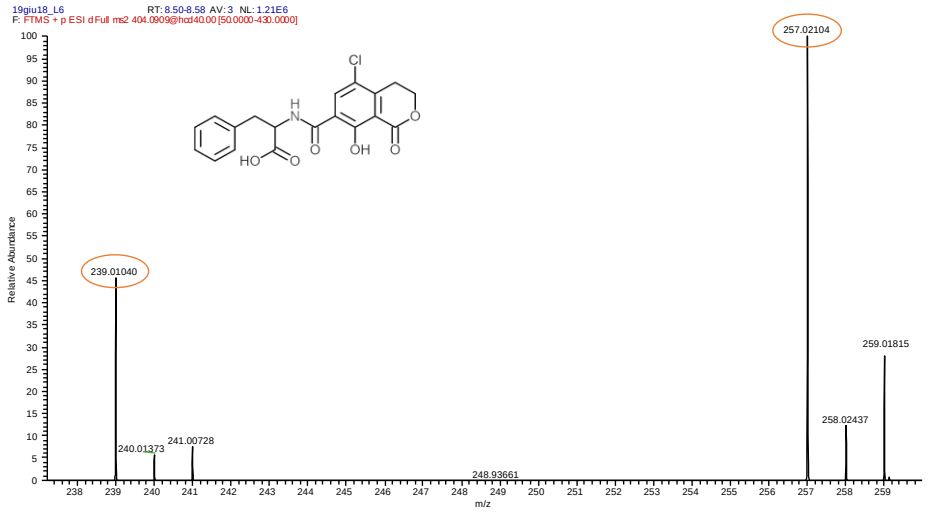


Figure 18. Data dependent mass spectrum (dd-MS^2) of OTA.

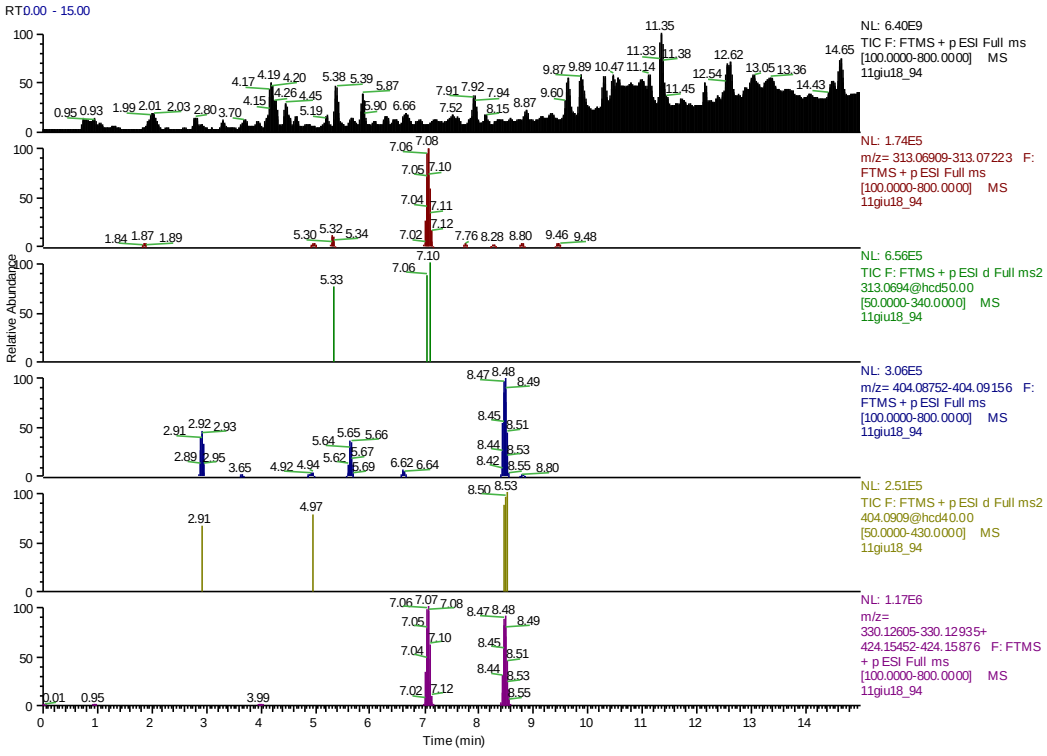


Figure 19. Chromatogram obtained by the injection of a serum sample naturally contaminated with AFB_1 and OTA. From the top, the TIC of the full scan in the range 100-800, AFB_1 peak followed by dd-MS^2 of AFB_1 fragments, OTA peak followed by dd-MS^2 of OTA fragments and the extracted ion chromatograms of AFM_1, U-[$^{13}C_{17}$]-AFB_1 and U-[$^{13}C_{20}$]-OTA, respectively.

3.2.6.3. Analytical quantification

For mycotoxins quantification an internal standard (ISTD) approach was adopted. The internal standard for AFB_1 and OTA was the ^{13}C isotope labelled molecule in which all carbon atoms are substituted by the stable isotope ^{13}C. The calibration curve was obtained by plotting the ratio (standard area/^{13}C area) *versus* the concentration expressed in pg/mL_{serum}. The calibration curve was obtained by fitting the data with a linear regression model based on least squares method. ISTD was applied to the sample prior to extraction allowing to correct for extraction efficiency and matrix effects.

3.3. Method validation

Identification criteria were set for all the analyzed mycotoxins. Linearity and LOD and LOQ of the analytical methods were assessed. Precision and trueness were assessed from repeated analyses on spiked blank urine and serum samples. Precision was evaluated by calculating the intermediate relative standard deviation (repeated analyses on different days), while trueness was estimated in terms of apparent recovery (R_A). Extraction efficiency (R_E) and matrix effect (SSE) were also evaluated during the validation process.

3.3.1. Identification criteria

According to the criteria reported in the DG SANTE guidance document on identification of mycotoxins in food and feed (SANTE/12089/2016), the retention time (RT) of the analyte in the sample extract should correspond to the average RT of the calibration standards measured in the same sequence with a tolerance of ±0.1 min. Moreover, for the ^{13}C-isotopically labelled analogue of the analyte (internal standard) added to the sample extract, the RT of the analyte should correspond to that of its labelled internal standard added to the pure solvent standard solution with a tolerance of ±0.05 min. For HRMS analysis identification is based on observation of the molecular ion (or, if not available, adducts) and at least one fragment that is specific for the selected analyte.

3.3.2. Linearity

Linearity of the method was evaluated from six points calibration curves injected in triplicate for three consecutive days, for each mycotoxin/matrix combination. For each calibration point a response ratio between response of target compound and response of internal standard was calculated and plotted against concentration. Regression lines were plotted applying a linear regression model based on least squares method.

The linearity was assessed by visual checking of the residual plot of response ratios (plotted in y-direction) versus the respective concentration levels (plotted in x-direction). The final estimated linearity model was verified using the lack-of-fit test (significance of the test with p_{value} below 0.05), to confirm that the selected regression and linearity were acceptable. Once visual checking of the residual and lack-of-fit test passed, the R squared coefficient was taken as a measure of linearity.

3.3.3. Limit of detection and quantification

The limit of detection (LOD) is defined as the smallest amount or concentration of analyte in the test sample that can be reliably distinguished from zero (Thompson, 2002).

The limit of quantification is the lowest concentration of analyte that can be determined with an acceptable repeatability and trueness (Thompson, 2002).

According to Wenzl (Wenzl, 2016), the variability of multiple analyses of representative matrix blank samples may be used to estimate the LOD of an analyte in the respective matrix. If blanks do not exist or if a signal cannot be obtained from

blank analysis, a pseudo-blank sample (blank sample spiked at low level) may be used instead. The spiked blanks approach was used for LOD and LOQ assessment, the spiked sample was analyzed in ten replicates under repeatability conditions. The variability, expressed as standard deviation, obtained for the ten analyses of spiked blanks was used for the estimation of the critical value of LOD. Calculation was carried out according to Equation A.

$$x_{LOD} = 3.9 * \frac{s_{y,b}}{b} \qquad \text{(A)}$$

Where $s_{y,b}$ is the standard deviation of the 10 replicates and b represents the slope of a dedicated calibration curve at concentration levels close to the expected LOD value.

According to Wenzl (Wenzl, 2016), the LOQ was estimated according to Equation B.

$$x_{LOQ} = 3.3 * x_{LOD} \qquad \text{(B)}$$

The LOQ values obtained with the theoretical calculation approache were included in the validation as the lowest concentration level.

3.3.4. Apparent recovery, matrix effect and extraction recovery

The validation was carried out on 5 different levels of contamination, including the calculated LOQ values, for each level triplicate analyses of spiked blank sample on 2 consecutive days were performed (3 + 3 = 6 spiked samples for each validated level). Due to the limited amount of blank sample, especially for serum, validation experiments were conducted with the aim of minimizing the amount of sample needed. For this purpose, blank samples were spiked at the beginning of the analytical procedure, while isotopically labelled internal standards were added before injection into LC-HRMS system. The obtained data were used for apparent recovery (R_A), matrix effect and extraction recovery (R_E) calculations and for precision assessment.

The **apparent recovery** is calculated as the ratio between the slope of the spiked sample curve, obtained from the spiked samples, and the slope of the calibration curve in pure solvent (Equation C). In this case, the curves were obtained considering the area and not the ratio with the labelled internal standard added for each mycotoxin. The R_A represents the influence of the whole analytical process (sample preparation + determination) on the signal and it is also referred to as overall or total recovery of a method. R_A was the parameter used for **trueness** evaluation.

$$R_A(\%) = \frac{slope_{spiked\ sample}}{slope_{pure\ solvent}} \times 100 \qquad \text{(C)}$$

The **matrix effect** was evaluated in terms of Signal Suppression/Enhancement (SSE) and it was calculated, according to Equation D, as the ratio between the mean area of the labelled internal standard (ISTD) in the spiked sample extracts and in the pure solvent standard solutions.

$$SSE\ (\%) = \frac{ISTD\ mean\ area_{extract}}{ISTD\ mean\ area_{pure\ solvent}} \times 100 \qquad \text{(D)}$$

The **extraction recovery**, accounting to incomplete extraction of the analyte from the matrix, was calculated from R_A and SSE, according to Equation E.

$$R_E\ (\%) = \frac{R_A}{SSE} \times 100 \qquad \text{(E)}$$

Precision was estimated in terms of intermediate precision as Relative Standard Deviation of repeatability (RSD_r).

4. RESULTS AND DISCUSSION

4.1. Method validation

4.1.1. Identification criteria

Identification criteria were met during validation for AFB_1, AFM_1 and OTA and were used for identification in the determination of each analyzed sample.

4.1.2. Linearity

Linearity was checked in the working range by the lack-of-fit test based on the analysis of variance (F test with $p_{value} < 0.05$) and the plot of the residual values randomly distributed around zero, confirming the linearity. During routine analytical sessions an $R^2>0.990$ was set as a criterion for calibration curve acceptability.

In Table 4 the calibration curve ranges, the amount of labelled internal standard added to each calibration level and the correlation coefficients obtained for each mycotoxin/matrix combination are reported. In Figure 20 the calibration curves obtained for AFB_1 and AFM_1 in urine with dilute&shoot and IAC clean-up method are reported. Calibration curves for AFB_1 and OTA in serum are reported in Figure 21.

Table 4. Calibration curve range, labelled internal standard concentration and correlation coefficients obtained for each mycotoxin/matrix combination are reported.

	Calibration curve range (pg/ml)			**Labelled standard (pg/ml)**			**R^2 (RSD)**		
	AFB_1	AFM_1	OTA	U-[$^{13}C_{17}$]-AFB_1	U-[$^{13}C_{17}$]-AFM_1	U-[$^{13}C_{20}$]-OTA	AFB_1	AFM_1	OTA
Urine – dilute&shoot	5 – 100	10 - 200	-	10	20	-	0.9965 (0.04)	0.9967 (0.20)	-
Urine – IAC clean-up	2.5 - 50	5 - 100	-	50	100	-	0.9973 (0.09)	0.9976 (0.10)	-
Serum	2.5 – 50	-	250 - 5000	50	-	2000	0.9963 (0.20)		0.9966 (0.20)

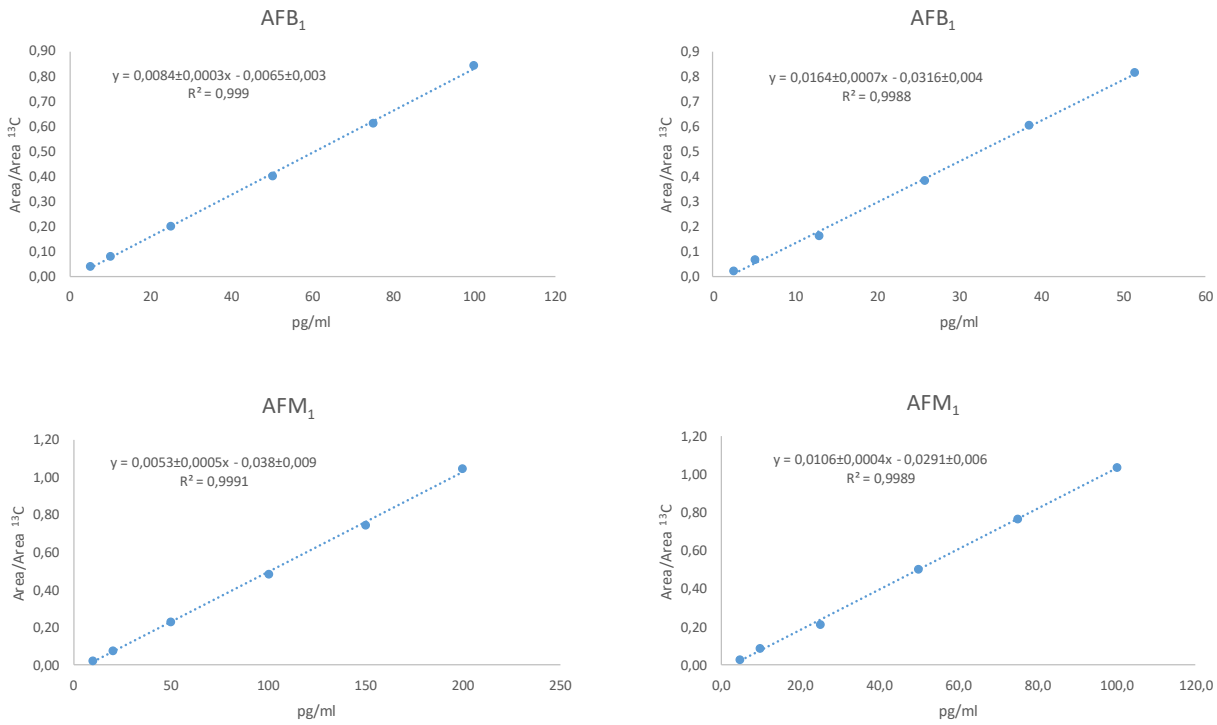


Figure 20. Calibration curves prepared for AFB_1 and AFM_1 determination in urine with dilute&shoot method (left side) and with IAC clean-up method (right side).

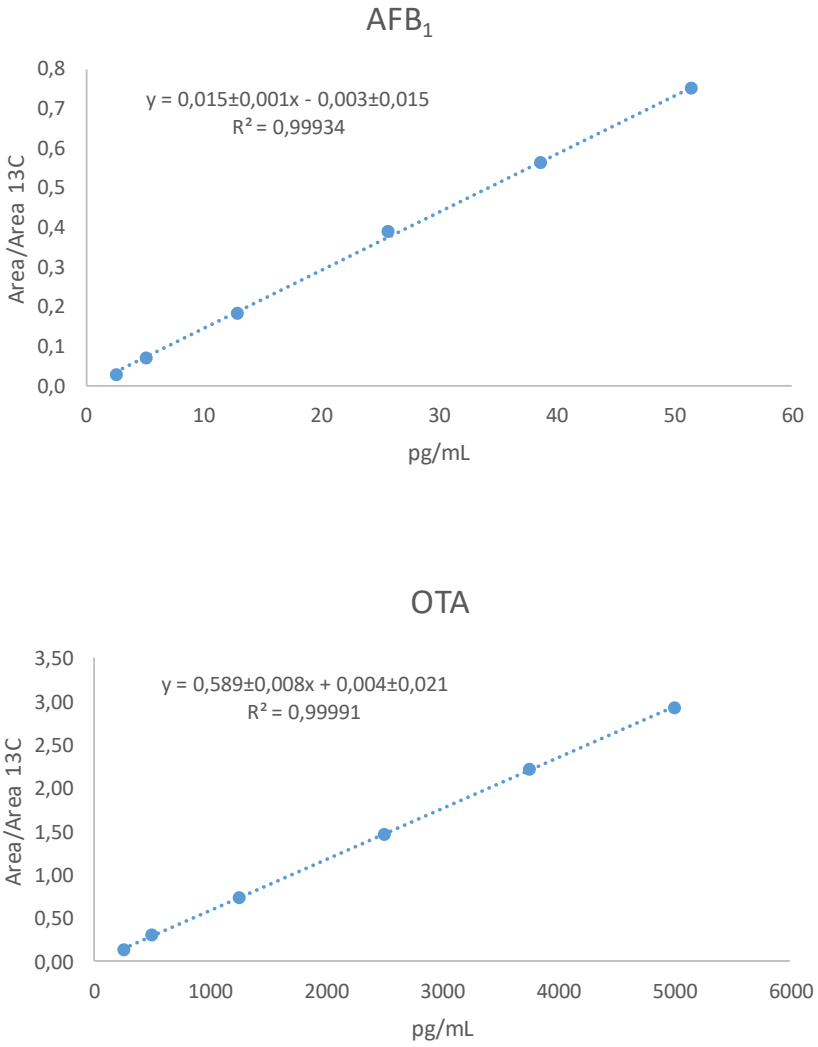


Figure 21. Calibration curves prepared for AFB_1 and OTA determination in serum.

4.1.3. Limit of detection and quantification

Limit of detection was assessed as described in section 3.3.3 by replicate analyses (n=10) of a spiked blank sample for both urine and serum. For quantification purpose, a 6 points calibration curves at concentration levels close to the expected LOD value were build, namely 2 – 20 pg/mL for AFB_1, 4 – 40 pg/mL for AFM_1 and 200 – 2000 pg/mL for OTA. As required, the upper concentration level of calibration standards to be used for the estimation of the value of b should not exceed 10 times the expected LOD.

The LOQ values were calculated from Equation B given in section 3.3.3. The values obtained from this theoretical calculation were included in the validation as the lowest concentration level.

4.1.4. Apparent recovery, matrix effect and extraction recovery

4.1.4.1. Urine

The performance characteristics of the urine methods are summarized in Table 5. Both methods may be applied for quantitative analysis of AFB_1 and AFM_1 as well as for the evaluation of presence/absence of the AFB_1-N^7-Guanine adduct. The dilute&shoot method is characterized by higher LOD and LOQ values when compared with the IAC clean-up method, but on the other hand the dilute&shoot approach is very quick and characterized by a conservative approach with respect to the sample, giving the possibility of a retrospective analysis on the acquired data. Due to the absence of a sample pretreatment only matrix effect, in terms of SSE, and precision, in terms of relative standard deviation (RSD_r), were evaluated during dilute&shoot method validation. SSE percentages for AFB_1 and AFM_1 are very close to 100% due to the dilution applied to the urine sample; method precision was assessed by performing 8 independent analyses at the LOQ level.

The IAC clean-up method was fully validated, trueness was evaluated in terms of apparent recovery while precision was assessed by repeatability RSD measures. The results, reported in Table 5, are considered satisfactory and the method fitting for the purpose. Although the IAC clean-up, which is a very selective approach, the influence of the matrix was evaluated. The percentages of SSE for AFB_1 and AFM_1 reveal that the influence of the matrix on the instrumental response is very limited. Anyway, the use of labelled internal standard for quantification always correct for the extraction efficiency and for the influence due to the presence of matrix in the ionization step.

Table 5. Performance characteristics obtained during validation for AFB_1 and AFM_1 in urine with dilute&shoot and IAC clean-up methods.

	Dilute&shoot method		**IAC clean-up method**	
	AFB_1	**AFM_1**	**AFB_1**	**AFM_1**
LOD (pg/ml_{urine})	20	40	0.8	1.5
LOQ (pg/ml_{urine})	50	100	2.5	5.0
Working range (pg/ml_{serum})	50.0 – 1000.0	100.0 – 2000.0	2.5 – 25.0	5.0 – 50.0
R_A (%)	-	-	101	98
R_E (%)	-	-	97	92
SSE (%)	82	111	104	107
RSD_r	8	11	6	12

4.1.4.2. Serum

The serum method allows the quantitative determination of AFB_1 and OTA and the evaluation of presence/absence of the AFB_1-Lysine adduct. The validation was conducted on 5 different levels of contamination, including LOQ. The results obtained during the validation process are reported in Table 6. The total RSD_r for AFB_1 and OTA are 11 and 9 respectively; apparent recovery is 55% for AFB_1 and 61% for OTA. While precision is in compliance with requirements established by this laboratory ($RSD_r \leq 20\%$), based on different reference standards (Commission Regulation 401/2006, Commission Decision 2002/657), recovery percentages are relatively low, even if, considering references standard for AFB_1 (Commission Regulation 401/2006, Commission Decision 2002/657) at these low levels and the complexity of the analyzed matrix, reasonably acceptable.

The serum method included a clean-up step with a QuEChERS, which allows a limited purification of the sample, however the matrix effect experienced results in a quite low signal suppression, especially for OTA, for which SSE falls in the range 90-120 where it is reported to be negligible (Malachová, 2014).

Table 6. Performance characteristics obtained during validation for AFB_1 and OTA in serum samples.

	AFB_1	**OTA**
LOD (pg/ml_{serum})	1.5	180
LOQ (pg/ml_{serum})	5	500
Working range (pg/ml_{serum})	5.0 – 50.0	500.0 – 5000.0
R_A (%)	55	61
R_E (%)	67	63
SSE (%)	82	96
RSD_r	11	9

4.2. Analytical results

4.2.1. Statistical analyses and Data handling - Left censored data

A statistical analysis was carried out to describe the analytical results dataset. The hypothesis of normality distribution (Shapiro–Wilk test) was refused, thus non-parametrical tests (which do not imply any distribution assumption) were used for the statistical treatment. All possible differences between concentration levels of mycotoxins in exposed and non-exposed groups were explored by a Wilcoxon rank-sum test. To assess the correlation between mycotoxin levels, a Spearman's rank correlation coefficient (or Spearman's rho) was used. All tests were conducted with a level of significance of 5%. Analyses were conducted by means of STATA14 software (Stata/IC 14.0, Copyright 1985–2015 StataCorp LP).

The LOQ levels were assessed during validation, and more specifically, for all the presented methods, the LOQ corresponded to the first validated contamination level, consistently with the established criteria of precision and trueness. LOD was estimated case by case in the analyzed samples, all samples below LOQ were considered positive reflecting the presence of the characteristic fragments in data dependent acquisition. Under the rigid identification criteria for analyte determination in urine and serum, namely the retention time criteria (RT±0.1 min with respect to the standard RT) and the presence of the precursor ion and at least one characteristic fragment for each considered analyte, it was decided to include and report also all the values below LOQ obtained by the interpolation of the calibration curve. Thus, values lower than LOQ were reported in the dataset as positive samples provided that the identification criteria were met.

The remaining results are to be considered as left censored data and for LB and UB mean calculation a substitution method was applied. In particular, for LB and UB calculation, zero and LOQ values were used respectively. The choice of LOQ instead of LOD in the substitution method is due to the fact that LOD was not unequivocally determined but was estimated case by case in the analyzed samples. Besides, the LOQ better represents the worst case within the LB and UB range, representing the optimistic (LB) or pessimistic (UB) scenario deriving from the sample analyses.

Due to variation in matrix composition, matrix effect tolerances were evaluated. An acceptance criterion was set for serum samples with respect to the R_A values calculated for each analyzed sample as the ration between the area of the labelled ISTD in the sample and the mean area of the labelled ISTD in pure solvent standard solutions used for the calibration curves. The samples with R_A<40% were excluded.

4.2.2. Urine sample analyses

The collected urine samples were analyzed first with the dilute&shoot method and none of the sample showed a measurable level of AFB_1 or AFM_1; also the adduct of AFB_1 with guanine was not detected.

To overcome the limitations coming from the detection limit threshold of the dilute&shoot method and verify that the negativity of the results could be caused by the level of LOD/LOQ declared, it was decided to set up and validate a method with lower LOQ. A purification step was introduced using an immunoaffinity column with the aim of cleaning and concentrating the urine sample. By using this

method for reprocessing the urine samples, AFB_1 and its adduct with guanine were not detected, AFM_1, instead, was found in 14 samples (12%) within the range 1.9-10.5 pg/mL_{urine}. The positive samples for AFM_1 < LOQ were in the range 1.9 - 4.7 pg/mL_{urine}. Only one sample showed a value above the LOQ (10.5 pg/mL_{urine}).

Table 7 summarizes the number and percentages of positive samples, maximum values found, and mean values (LB-UB, and positive). To note that when values reported for AFM_1 are below the LOQ they shall be considered as affected by a standard uncertainty higher than 25%, which was the performance criteria set for maximum standard uncertainty for the LOQ.

For the mean calculations, the substitution method was applied. The LB mean value was obtained substituting the zero value to all the samples where AFM_1 was not detected; the upper-bound (UB) mean value was obtained substituting the LOQ value (5 pg/mL_{urine}) to all the samples where AFM_1 was not detected. These two mean values reflect the optimistic and pessimistic scenario range of possible mean values.

Figure 22 shows the data trend for AFM_1 in urine for both groups, Monday and Friday sampling. In the left side UB substitution method was applied, due to the high number of non detected (87%), box plot is flattened to the mean value (5 pg/mL_{urine}). In the right side the box plot reports all the positive values. The band inside the box is the second quartile (P50, median). Dots are suspected outliers. Whiskers are set from minimum to maximum value. The bottom and the top of the box are the first and third quartiles (P25 and P75).

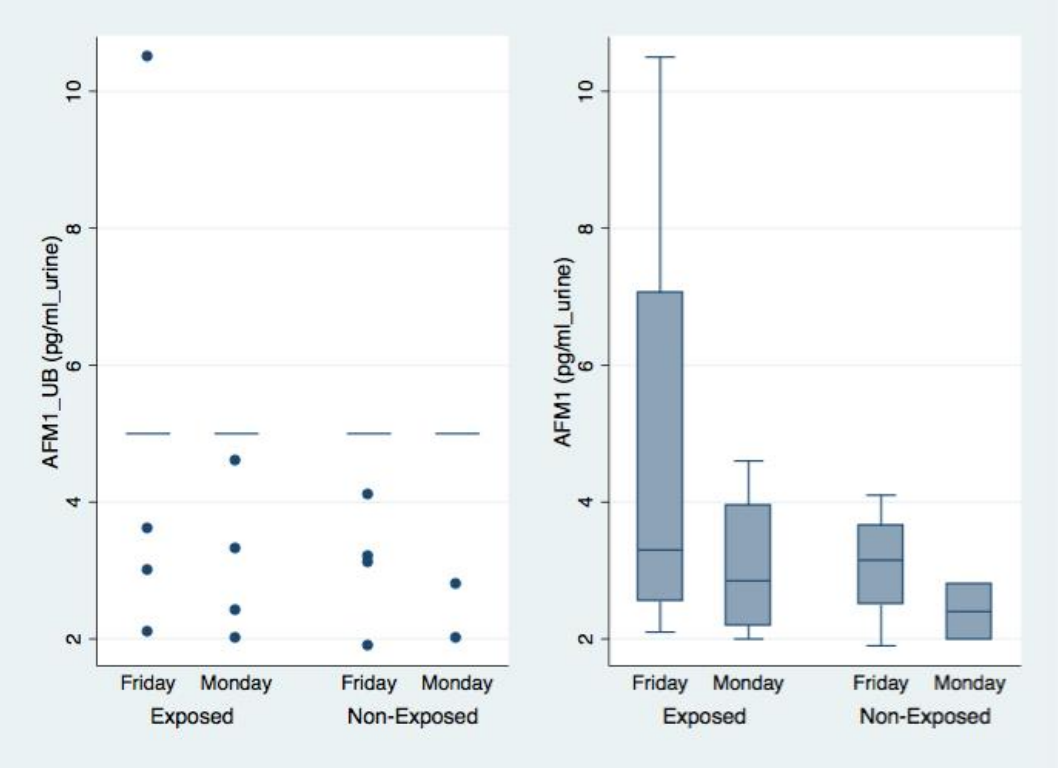
Table 7. Results of AFB_1 and AFM_1 urinary biomarkers of worker and control groups.

Exposed workers group	**AFB_1**	**AFM_1**
Monday and Friday; subjects (n=63)		
Positive[a] (n)	0	8
Positive (%)	0	13
Max[b] (pg/mL_{urine})	-	10.5
Mean (LB-UB) (pg/mL_{urine})	-	0.5-4.9
Mean positive (pg/mL_{urine})	-	3.9
Monday; subjects (n=32)		
Positive (n)	0	4
Positive (%)	0	13
Max (pg/mL_{urine})	-	4.6[c]
Mean (LB-UB) (pg/mL_{urine})	-	0.4-4.48
Mean positive (pg/mL_{urine})	-	3.1
Friday; subjects (n=31)		
Positive (n)	0	4
Positive (%)	0	13
Max (pg/mL_{urine})	-	10.5
Mean (LB-UB) (pg/mL_{urine})	-	0.6-5.0
Mean positive (pg/mL_{urine})	-	4.8
Control group; subjects		
Monday and Friday; subjects (n=57)		
Positive (n)	0	6
Positive (%)	0	11
Max (pg/mL_{urine})	-	4.1[c]
Mean (LB-UB) (pg/mL_{urine})	-	0.3-4.8
Mean positive (pg/mL_{urine})	-	2.9
Monday; subjects (n=29)		
Positive (n)	0	2
Positive (%)	0	7
Max (pg/mL_{urine})	-	2.8[c]
Mean (LB-UB) (pg/mL_{urine})	-	0.2-4.8
Mean positive (pg/mL_{urine})	-	2.4
Friday; subjects (n=28)		
Positive (n)	0	4
Positive (%)	0	14
Max (pg/mL_{urine})	-	4.1[c]
Mean (LB-UB) (pg/mL_{urine})	-	0.4-4.7
Mean positive (pg/mL_{urine})	-	3.1

[a)]Positive: values above LOD

[b)]Max: maximum value

[c)]Value below the LOQ



Left side, mean UB values; right side, mean positive values. The horizontal band (inside the box) is the second quartile (P50, median). Dots are suspected outliers. Whiskers are set from minimum to maximum value. The bottom and the top of the box are the first and third quartiles (P25 and P75).

Figure 22. Urine data graphs of AFM_1 in exposed and non-exposed workers, Monday and Friday deliveries.

No statistical difference was observed between Monday and Friday samples in each group (exposed and non-exposed workers). To note that two individuals of the exposed workers group showed AFM_1 in both Monday and Friday deliveries with a concentration level of 3.3 and 3.0 pg/mL_{urine} and 4.6 and 10.5 pg/mL_{urine} as Monday and Friday values for each individual, respectively.

Further statistical analyses were performed merging data of Monday and Friday (63 analyses for exposed workers group and 57 for non-exposed workers group). Examining in depth the samples within and between the groups, it was highlighted that: i) eight samples (13%) resulted positive in the workers group where the highest contaminated sample was found (10.5 pg/mL$_{urine}$); ii) six samples (11%) were positive in the control group, the higher detected value was 4.1 pg/mL$_{urine}$. In order to find differences among the positive values found in workers and control group, a Wilcoxon rank-sum test was performed but no statistical significances were highlighted. Even exploring the two days of urine delivery (i.e. sampling on Monday and Friday), no differences were highlighted.

The absence of a statistical difference when the mean values for workers and control groups are compared suggests that in this specific setting, no professional exposure occurs. Moreover, taking into account the very low level of AFM_1 in the collected urine samples, also the contribution from the diet to the overall exposure is to be considered negligible.

In a previous study (Ferri, 2017), conducted in 2013 in the same Italian feed plant, higher values of AFM_1 content in urine samples were reported, namely mean values of 35 and 27 pg/mL$_{urine}$ for workers and controls group, respectively. However, also in this case, no statistical difference was highlighted when the two groups were compared, suggesting that the exposure only accounts for the contribution due to the diet. The differences in contamination levels detected in the two studies may be explained by taking into account the influence that weather conditions have on mycotoxin production. In 2012, North Italy experienced an extremely dry season,

thus during the 2012-2013 harvest, food with higher AFB_1 level may be released on the market. Also the presence of AFB_1 in feed may results in a AFM_1 contamination in cow milk, source of AFM1 exposure also for humans. However, also in this first study the AFM_1 levels detected were low depicting a safe scenario in terms of AFB_1 exposure.

4.2.3. Serum sample analyses

Serum samples were analyzed for AFB_1 and the presence/absence of AFB_1-Lysine adduct. Due to the versatility of the analytical method, free OTA was also evaluated. Due to serum sample composition variability, the labelled internal standard was added at the beginning before sample extraction in order to take into account the total recovery (R_A) due to extraction recovery and SSE contributions. In some cases, the influence of the matrix on the method was very strong leading to R_A value considered unacceptable. More specifically for this purpose, inclusion/exclusion criteria for accepting values was established, and it was decided to exclude sample results showing R_A lower than 40%.

The obtained results are presented in Table 8. The percentage of positive samples reported in the table is calculated on the number of samples retained after the exclusion due to unacceptable R_A, namely 62 and 59 exposed worker samples for AFB_1 and OTA, respectively, and 52 and 50 control samples for AFB_1 and OTA, respectively. None of the analyzed samples showed the presence of AFB_1-Lysine adduct; however, it should be reminded that it was not possible to define the

concentration level of the synthetized product, therefore the adduct absence may be due to high LOQ value of the method.

The samples with OTA < LOQ were in the range 0.05 - 0.49 ng/mL_{serum}, no positive samples for AFB_1 were < LOQ. To note that, in Table 8, when values reported for OTA are below the LOQ they shall be considered as affected by a standard uncertainty higher than 25%, which was the performance criteria set for maximum standard uncertainty for the LOQ.

The number and percentages of positive samples, maximum values found, and mean values (LB-UB, and positive) are reported. For the mean calculations, the substitution method was used. The LB mean value was obtained substituting the zero value to all the samples where AFB_1 was not detected; the upper-bound (UB) mean value was obtained substituting the LOQ value (5 pg/mL_{serum}) to all the samples where AFB_1 was not detected. These two mean values reflect the optimistic and pessimistic scenario range of possible mean values.

In the same way as urine statistical assessment, no differences were observed either comparing AFB_1 Monday and Friday values in each group (exposed and non-exposed workers), neither comparing values between exposed and non-exposed workers group (Monday and Friday merged) (Wilcoxon rank-sum test). To note that two individuals of the exposed workers group showed AFB_1 in serum (2.4 and 4.6 pg/mL_{serum}) and AFM_1 in urine (289.8 and 154.9 pg/mL_{urine}) both toxins in Monday deliveries.

The same consideration made for urine analytical results can be done for the results obtained for serum. The low incidence of aflatoxins in serum cannot be attributable

to occupational exposure and reveals a safe scenario also for dietary exposure by AFB_1.

The 100% of the analyzed samples revealed the presence of OTA with 33% of the samples showing a concentration higher than the LOQ (0.5 ng/mL_{serum}) and the remaining 67% with a signal below the LOQ.

For OTA, taking into account that in this context (corn-based feedstuffs company) this mycotoxin is not relevant for workers exposure, few considerations are done after scrutiny of the samples within and between the groups: i) no statistical differences were highlighted between the average levels of exposed and control groups; ii) no statistical differences were highlighted between Monday and Friday OTA levels; iii) a correlation (ρ= 0.48 p= 0.0005) was found between Monday and Friday OTA levels, without group distinction, which emphasizes the consistency of the OTA content in the serum coherently with the persistence of OTA in human beings with a blood half-life of 35 days after a single oral dosage due to unfavorable elimination toxicokinetics (Petzinger, 2000).

Figure 23 and 24 shows the data trend for AFB_1 and OTA in serum for both groups, Monday and Friday sampling. In Figure 23, UB substitution method was applied and due to the high number of non detected (90%), box plot is flattened to the mean value (5 pg/mL_{serum}).

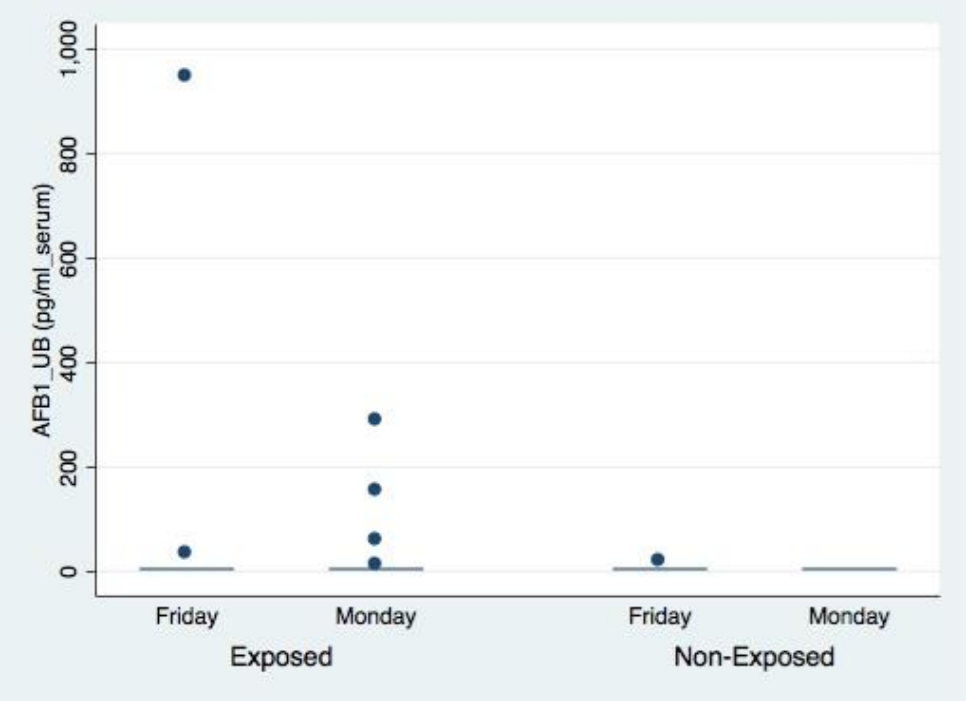
Table 8. Results of AFB_1 and OTA serum biomarkers of worker and control groups.

Exposed workers group	**AFB_1**	**OTA**
Monday and Friday; subjects (n=63)		
Excluded (n)	1	4
Positive[a] (n)	6	59
Positive (%)	9.7	100
Max[b] (pg/mL_{serum})	947.4	3700
Mean (LB-UB) (pg/mL_{serum})	24.2-28.7	-
Mean positive (pg/mL_{serum})	249.9	600
Median (pg/mL_{serum})	-	380[c]
Monday; subjects (n=32)		
Excluded (n)	1	3
Positive[a] (n)	4	29
Positive (%)	12.9	100
Max[b] (pg/mL_{serum})	289.8	2880
Mean (LB-UB) (pg/mL_{serum})	16.7-21.0	-
Mean positive (pg/mL_{serum})	249.9	600
Median (pg/mL_{serum})	-	360
Friday; subjects (n=31)		
Excluded (n)	-	1
Positive[a] (n)	2	30
Positive (%)	6.5	100
Max[b] (pg/mL_{serum})	947.4	3700
Mean (LB-UB) (pg/mL_{serum})	31.7-36.4	-
Mean positive (pg/mL_{serum})	491.8	600
Median (pg/mL_{serum})	-	380
Control group; subjects		
Monday and Friday; subjects (n=55)		
Excluded (n)	3	5
Positive[a] (n)	1	50
Positive (%)	1.9	100
Max[b] (pg/mL_{serum})	19.7	6450
Mean (LB-UB) (pg/mL_{serum})	0.4-5.3	-
Mean positive (pg/mL_{serum})	-	600
Median (pg/mL_{serum})	-	370
Monday; subjects (n=28)		
Excluded (n)	1	2
Positive[a] (n)	0	26
Positive (%)	-	100
Max[b] (pg/mL_{serum})	-	2330
Mean (LB-UB) (pg/mL_{serum})	-	-
Mean positive (pg/mL_{serum})	-	530
Median (pg/mL_{serum})	-	450
Friday; subjects (n=27)		
Excluded (n)	2	3
Positive[a] (n)	1	24
Positive (%)	4	100
Max[b] (pg/mL_{serum})	19.7	6450
Mean (LB-UB) (pg/mL_{serum})	0.8-5.6	-
Mean positive (pg/mL_{serum})	-	680
Median (pg/mL_{serum})	-	350

[a)]Positive: values above LOD

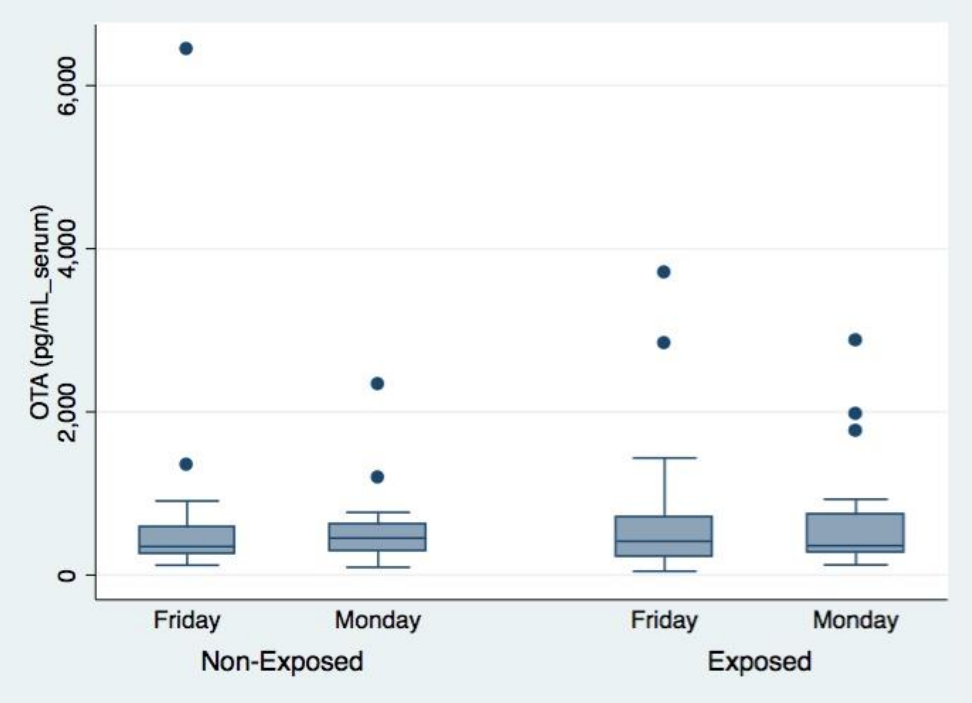
[b)]Max: maximum value

[c)]Value below the LOQ



The horizontal band inside the box is the second quartile (P50, median). Dots are suspected outliers. Whiskers are set from minimum to maximum value. The bottom and the top of the box are the first and third quartiles (P25 and P75).

Figure 23. Serum data graphs of AFB_1 in exposed and non-exposed workers, Monday and Friday deliveries.



The horizontal band inside the box is the second quartile (P50, median). Dots are suspected outliers. Whiskers are set from minimum to maximum value. The bottom and the top of the box are the first and third quartiles (P25 and P75).

Figure 24. Serum data graphs of OTA in exposed and non-exposed workers, Monday and Friday deliveries.

4.3. Estimated daily intake

The estimation of the daily intake (EDI) was performed only for OTA that accounts for a 100% of positive results. The EDIs are calculated as individual value, and compared to the reported TDI in order to evaluate the possible health concern due to this level of exposure.

Individual daily intakes were calculated starting from OTA level measured in serum samples. The daily intake of OTA was calculated on the basis of serum toxin levels using the Klaassen equation:

$$k_0 = \frac{Cl_p \times C_p}{A} \qquad \text{(F)}$$

Where:

k_0 is the daily intake (ng/kg bw/day)

Cl_p is the plasma clearance (mL/kg bw/day)

C_p is the serum concentration of ochratoxin A (ng/mL_{serum})

A is the bioavailability of the toxin

For A and Cl_p assumptions are made (Duarte, 2011): bioavailability (A), i.e., fraction of OTA taken up, is considered for most animals around 50% (Hagelberg, 1989); plasma clearance (Cl_p,) considers only renal filtration rates, which might underestimate the plasma clearance, since no other than renal filtration is contemplated (Breitholtz, 1991). Two main values exist for the calculation of human renal filtration rate, and so two major versions exist for the same equation

(Miraglia, 1996; Gilbert, 2001; Scott, 2005). The first value (C_p = 0.67 mL/kg·bw/day) was calculated from the glomerular filtration rate of inulin (0.033 mL/min) and the free fraction of OTA, 0.02% (Hagelberg, 1989). The second value (C_p = 0.99 mL/kg·bw/day) resulted from a study with a single human volunteer that ingested ^{3}H-labeled OTA, which allowed the calculation of renal clearance of OTA as 0.048 mL/min referred to a 70 kg person (Schlatter, 1996).

In this study, calculations were made with Cl_p = 0.99 mL/kg·bw/day, according to Schlatter et al. (1996) and A = 0.5 considering a 50% of OTA bioavailability (Hagelberg, 1989). Although the Equation F is referred to OTA content in plasma, according to Palli et al. (1999), it was assumed that serum specimen concentration might be approximated to plasma concentrations.

In 2006, EFSA established for OTA a Tolerable Weekly Intake (TWI) of 120 ng/kg bw/week, equivalent to a Tolerable Daily Intake (TDI) of 17.14 ng/kg bw (EFSA, 2006). In 2007, JECFA reconfirmed the established value of 100 ng/kg·bw/week based on pig nephrotoxicity, corresponding to a TDI of 14.28 ng/kg·bw/day. More recently, Kuiper-Goodman et al (2010) reevaluated the TDI as 4 ng/kg·bw/day.

In Figure 25 the EDIs are reported for workers group Monday-Friday (in red and orange, respectively) and for controls group Monday-Friday (in blue and light blue, respectively); the red line is the EFSA TDI while and the green line represents the TDI value reevaluated by Kuiper-Goodman et al. (2010).

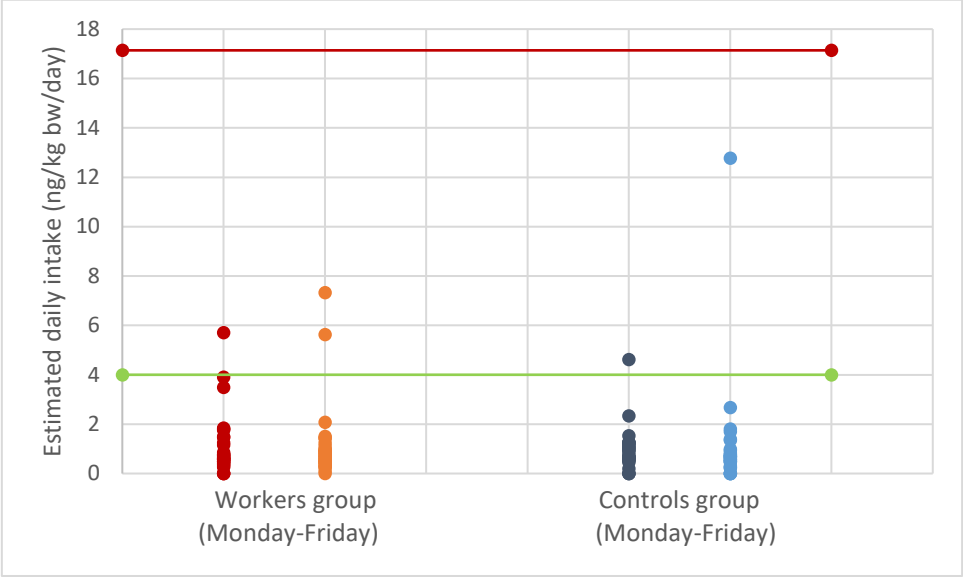


Figure 25. Individual OTA daily intake calculated for Workers group Monday-Friday (in red and orange, respectively) and for Controls group Monday-Friday (in blue and light blue). The red line represents the EFSA TDI value (17.14 ng/kg bw/day) and the green line represents a reevaluated TDI at 4 ng/kg bw/day.

From Figure 25 is observable that the EDI values are very low when compared with the EFSA TDI, with the highest value estimated being 12.77 ng/kg bw/day related to the Friday delivery of a volunteer from the controls group. When the revised TDI at 4 ng/kg bw/day is considered, 5 subjects showed an EDI above that threshold value, 3 results from the workers group, one sampled on Monday (5.70 ng/kg bw/day) and two on Friday (7.32 and 5.62 ng/kg bw/day), and 2 results from controls group, from Monday (4.61 ng/kg bw/day) and Friday (12.77 ng/kg bw/day) deliveries. Moreover, the EDIs exceeding the 4 ng/kg bw/day, for the workers group on Monday and Friday, are from the same subjects, namely 5.70 – 5.62 and 7.32 – 3.9 ng/kg bw/day). In total, the 6% of the EDIs exceeded the reevaluated TDI.

The estimated daily intakes were then compared with the Italian daily intake reported in the SCOOP Task "Assessment of dietary intake of ochratoxin A by the population of European Union Members States" (SCOOP Task, 202)elaborated in 2002 (Table 9). For this purpose, a single EDI mean value was calculated considering that no statistical differences were observed between workers and controls group and between Monday and Friday deliveries (1.19 ng/kg bw/day). The SCOOP Task reports the daily intake estimated as dietary intake (1.13 ng/kg bw/day) and via biomarkers approach (1.16 ng/kg bw/day). The EDIs reported in the SCOOP Task, estimated via traditional exposure calculations as dietary intake, or via biomonitoring approach, overlap with the mean value of the volunteers enrolled in this study, confirming that the estimated exposure is mainly due to the diet.

A previous work published by Brera et al. (2002) reports the OTA levels in serum collected from Italian volunteers working in cocoa, coffee and spices processing plants located in Tuscany, the derived EDIs, reported in Table 9, are lower but in line with the discussed values.

The obtained results were also compared with the values obtained in a recent publication originally aiming at assessing professional exposure that, in agreement with this study, concluded that the serum OTA levels are to be only attributable to the dietary intake (Viegas, 2018b). Moreover, the mean value reported for the enrolled population in the work of Viegas et al. is very close to the EDI presented in this study; also the ranges of OTA levels for both studies are in perfect agreement, confirming the primary role of the diet for OTA exposure (Table 9).

Table 9. Comparison of different estimated daily intakes.

	This study	SCOOP Task Biomarker approach[a]	SCOOP Task Dietary intake approach[a]	Italian workers[b] (Brera, 2002)	Portuguese waste workers[c] (Viegas, 2018)
EDI (ng/kg bw/day)	1.19	1.16	1.13	1.05	1.99
Range (ng/kg bw/day)	0.99-12.77	-	-	1.86-6.49	0.87-11.98
Median (ng/kg bw/day)	0.73	-	-	-	1.50

[a]EDIs calculated for Italian total population (SCOOP Task 3.2.7. 2002)

[b]EDIs calculated for Italian volunteers working in cocoa, coffee and spices processing plants (Brera, 2002)

[c]EDIs calculated for volunteers working in on Portuguese waste management setting (Viegas, 2018)

5. CONCLUSIONS

The presented study aims to explore the validity of the biomonitoring studies as a tool to investigate the intake of mycotoxins in population groups such as workers operating in risky workplaces. The study also intends to contribute to obtain a more comprehensive and updated dataset. Moreover, the work aimed to set up and validate suitable methods for the determination of the selected mycotoxins and their metabolites in specimens.

The study was conducted on two groups of population, the exposed workers groups that includes staffs working in an Italian feed plant, and a control group composed by administrative employees (non-exposed). Urine and serum samples were collected for the determination of mycotoxins, AFB_1, AFM_1 and AFB_1-N^7-Guanine adduct were analyzed in urine samples, while AFB_1, AFB_1-Lysine adduct and OTA were analyzed in serum samples.

In conclusion, no statistical difference was observed between workers and controls group for urine and serum samples revealing that no professional exposure to AFs and OTA occurred for the enrolled volunteers.

Regarding AFs results, the low positive samples in terms of both, prevalence and concentration levels, confirm that there is not a concern for public health deriving from the diet.

Regarding OTA analyses in serum, the 100% of the analyzed samples were positive. In particular, the 33% of the serum samples were above the LOQ value, however, it was decided, under the rigid identification criteria for analyte determination, to include and report also all the values below LOQ. The data

obtained for OTA were used for estimate the individual daily intake according to Klaassen equation. The comparison of the obtained results with TDI reported values reveals that the estimated daily intakes are far below the EFSA TDI (17.17 ng/kg bw/day) with the highest contaminated sample being 12.77 ng/kg/bw/day. Also the comparison with previous exposure studies related to Italian population referring to total population or workers, showed a general agreement in terms of estimated daily intake.

From the obtained results, it can be concluded that in the investigated occupational setting no professional exposure to OTA was experienced by the workers. Nevertheless, further studies are needed to also explore other settings where temperature and humidity conditions, together with indoors settings, for instance stables, might promote an occupational exposure via inhalation or dermal contact. Moreover, an EFSA opinion, reviewing the OTA TDI, has been requested by the European Commission and may require new evaluation of the obtained results.

In this study, different methods have been set and fully validated for the analysis of urine and serum with very low LOD/LOQ, also taking into consideration the presence of urine and serum AFB_1 adducts (N^7-Guanine and Lysine adducts, respectively). The availability of suitable method is of crucial importance for a reliable assessment of exposure. A future need is undoubtedly, the organization of inter-laboratory studies with the aim of completing the validation process with a comparison study and harmonizing the analytical procedures to be applied for biological fluid analyses.

Acknowledgements

This research was funded by EFSA: GP/EFSA/AFSCO/2017/05PERFORMANCE: International Conference “The Burden of Mycotoxins on animal and human health” and Research Project “Biomonitoring data as a tool for assessing aflatoxin B1 exposure of workers – BIODAF”. Sole responsibility lies with the author and the Authority is not responsible for any use that may be made of the information contained therein.

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