Optimization and validation of a LC-HRMS method for aflatoxins determination in urine samples

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Abstract
Mycotoxins’ exposure by inhalation and/or dermal contact can occur in different branches of industry especially where heavily dusty settings are present and the handling of dusty commodities is performed. This study aims to explore the possible contribution of the occupational exposure to aflatoxins by analysing urine samples for the presence of aflatoxins B1 and M1 and aflatoxin B1-N7-guanine adduct. The study was conducted in 2017 on two groups of volunteers, the workers group, composed by personnel employed in an Italian feed plant (n = 32), and a control group (n = 29), composed by the administrative employees of the same feed plant; a total of 120 urine samples were collected and analysed. A screening method and a quantitative method with high-resolution mass spectrometry determination were developed and fully validated. Limits of detections were 0.8 and 1.5 pg/mLurine for aflatoxin B1 and M1, respectively. No quantitative determination was possible for the adduct aflatoxin B1-N7-guanine. Aflatoxin B1 and its adduct were not detected in the analysed samples, and aflatoxin M1, instead, was found in 14 samples (12%) within the range 1.9–10.5 pg/mLurine. Only one sample showed a value above the limit of quantification (10.5 pg/mL urine). The absence of a statistical difference between the mean values for workers and the control group which were compared suggests that in this specific setting, no professional exposure occurs. Furthermore, considering the very low level of aflatoxin M1 in the collected urine samples, the contribution from the diet to the overall exposure is to be considered negligible.

Keywords Biomonitoring · Biomarker · Mycotoxin · Aflatoxin · Metabolites · LC-Orbitrap · LC-HRMS

Introduction
Among xenobiotics, mycotoxins, secondary metabolites of fungal origin, are the most harmful hazards with high toxic potency and recognized adverse impacts on human and animal health. More than 500 mycotoxins are known, but scientific studies focus on those that exert carcinogenic and/or toxic activity, and only few of them are regulated worldwide (Stein and Bulboaca 2017; CAST 2003; FAO 2004). Among mycotoxins, aflatoxins (AFs) represent one of the most concerning class of chemical compounds with a focus of interest on aflatoxin B1 (AFB1) that, due to its acute and chronic toxic effects, have raised the interest of the scientific community. The primary target organ affected by aflatoxin B1 exposure is the liver, and several epidemiological studies related to AFB1 exposure to cellular hepatocarcinoma report it as one of the major cause of cancer-related deaths in different parts of the world (Wild and Turner 2002). AFB1 is a genotoxic and carcinogenic substance, classified under group 1 by the International Agency for Research on Cancer (IARC 1993). AFs can occur in crops at preharvest, harvest and postharvest stages as a result of different co-occurring environmental conditions and poor management practices (handling and storage). The expected global warming of +2 °C is likely to cause a sensible climate change leading to conducive environmental conditions for AFs production in Northern Europe, where currently no occurrence is significantly present (Battilani et al. 2016). Therefore, validating new methods for AFs...
determination becomes particularly relevant to be applied in newly exposed geographical regions.

The most common route of exposure to mycotoxins is the ingestion through the diet due to the consumption of directly or indirectly contaminated food. Furthermore, humans and animals can also be exposed to mycotoxins through inhalation and/or dermal contact with contaminated dusts (Brera et al. 2002; Doi and Uetsuka 2014; Viegas et al. 2014, 2017). Several studies reported a higher prevalence of lung carcinogenesis and bronchus and trachea tumours in workers exposed to aflatoxin-contaminated dusts (McLaughlin et al. 1987; Olsen et al. 1988; Ghosh et al. 1997; Saad-Hussein et al. 2013, 2014), especially in branches of industry where the storage, loading, milling and handling of dusty commodities (such as grains, feed, spices, coffee, etc.) is performed. Due to their severe toxicological implications, exposure to aflatoxins 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 biomonitoring studies. The overall metabolic pathway of AFB1 is quite complex and corresponds to the formation of a number of metabolites that could be associated not only to the dose of the parent mycotoxin but also to the biological response to the exposure and to the degree of individual sensitivity to adsorption and metabolism of the toxic agent (Groopman 1994). Validated exposure biomarkers for AFB1 (urinary aflatoxin M1, AFB1-N7-guanine) were established almost 20 years ago (Groopman et al. 1993), they were critical in confirming aflatoxins as potent liver carcinogens, and more importantly, they are being used to assess the effectiveness of intervention strategies (Cramer and Humpf 2017; Turner et al. 2012).

Biomonitoring studies have been increased over the last 8 years. In a recent publication, Viegas (Viegas et al. 2018) reviewed the use of biomonitoring in assessing occupational exposure to mycotoxin in different settings, and 58% of the reviewed works assessed aflatoxins exposure. Despite the impossibility to distinguish between dietary and air-dust contamination, the literature review clearly showed that, under certain circumstances, workers were significantly more exposed than the control group (Malik et al. 2014; Saad-Hussein et al. 2014; Viegas et al. 2016). In Italy, a first study on occupational exposure to aflatoxins was conducted in 2014 in two feed companies, to assess if workers occupied in dusty indoor settings were differently exposed than workers occupied in administrative units (control group) (Berri et al. 2017). To monitor the situation and to assess the effect of new agricultural season, the same scheme of the study was replicated in 2017 within a different analytical framework, where also the guanine metabolite was included.

The present study aims to explore the role of the occupational exposure to aflatoxins by analysing urine samples to assess the presence of aflatoxins B1 and M1 and aflatoxin B1-N7-guanine adduct in a group of workers, operating in risky workplaces, and a control group. The group of volunteer workers, operating in a setting of the feed sector, potentially exposed to mycotoxins through the inhalation of contaminated dust and/or by dermal contact, and a control group, composed by administrative employees working on the same feed plant, were enrolled in the study.

Aflatoxins determination was performed by a high-resolution mass spectrometry (LC-HRMS) technique. For sample preparation, a dilute and shoot method and a quantitative method based on immunoaffinity column purification step were developed and fully validated. Moreover, due to the unavailability of commercial standard of AFB1-N7-guanine, the adduct was synthetized and used for the method set-up and for qualitative analysis (presence/absence) in the collected samples.

Materials and methods

Chemicals and reagents

Chemicals and solvents used for sample preparation were LC-MS grade. Methanol, formic acid and LC-MS grade water were purchased from Fisher Scientific (Milano, Italy), and AFB1 from Aspergillus flavus (purity ≥ 98%) was from Sigma-Aldrich (Darmstadt, Germany). The analytical reference standard of AFM1 was purchased as stock solution (0.5 μg/mL in acetonitrile) from Biopure® (Tulln, Austria). The isotopically labelled internal standards U-[13C17]-AFB1 (99.3% 13C) and U-[13C17]-AFM1 (98.3% 13C) were also purchased as acetonitrile solution (0.5 μg/mL) from Biopure® (Tulln, Austria). The concentration reported in the certificate accompanying the reference standard purchased as solution was considered for quantification purpose. The AFB1 powder was reconstituted with 100% ACN, and the concentration was assessed by molar absorbance value following the procedure reported in the official Methods of Analysis of AOAC (AOAC 2005). The AFB1-N7-guanine adduct was not commercially available at the moment of the study and was synthetized as reported below.

AFB1-N7-guanine adduct synthesis and identification

The synthesis was conducted accordingly with Vidyasagar et al. (1997) as follows: meta-chloroperboxbenzoic acid (MCPBA), 20 mg in 4 mL of dichloromethane, was washed with 100 mM mmol/L phosphate buffer, pH 7.4 (4 mL × 4). The resulting MCPBA solution was passed through anhydrous sodium sulphate to remove residual water. AFB1 (0.64 μmol) was dissolved in 250 μL of dichloromethane and was converted to AFB1–8,9-epoxide by addition of 250 μL of the above MCPBA solution (4 μmol) and 500 μL of 100 mM mmol/L 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. About 0.32 μmol of guanine, previously dissolved in 0.1 mol/L HCl, were added to 500 μL of 100 mM mmol/L phosphate buffer and 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 AFB1–8,9-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 × 3 times). The adduct identification was based on the observation of the molecular ion and at least one fragment specific for the analyte after injection in the LC-HRMS system, according to the guidance document on identification of mycotoxins in food and feed (EC 2016). Due to the difficulties in assessing the concentration level of the synthetized adduct, the diluted buffer fraction was used for testing the IAC cross reactivity during method development and for a qualitative evaluation of presence/absence in the collected urine samples.

Study design

The investigation was conducted in the same feedstuff plant involved in the first study previously published by Ferri et al. (2017). This second study was conducted within the framework of a larger project entitled “Biomonitoring data as a tool for assessing aflatoxin B1 exposure of workers – BIODAF” supported by EFSA (July 2017–June 2018). The project focused on aflatoxins and took into consideration urine and serum samples’ collection and analysis. Two countries, Italy and Portugal, were involved in this study. The present paper reports the results obtained from the Italian urine analyses.

Two groups of volunteers were enrolled, the “workers 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”, which included employees of the same company but designated to perform other activities considered not risky for the absence of contaminated environmental dusts. The samples were collected on Monday and Friday morning in one working week. Monday was chosen since it reflects a situation characterized by a preceding 2-days washing period, and Friday was selected with the aim to verify a possible accumulation of AFs and consequent intake over the week of sampling. The urine was collected in the morning and delivered to the medical staff before starting the morning shift. A total of 61 male volunteers were enrolled (32 workers and 29 controls). The collected 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.

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. All urine donors were informed about the purpose of the study, and a formal consent was individually signed prior to inclusion in the study.

Sample preparation

Dilute and shoot sample preparation

Before analysis, all urine samples were equilibrated to room temperature and homogenized by shaking thoroughly. Aliquots of 100 μL urine were mixed with 860 μL of H2O LC-MS grade; for quantification purpose, 20 μL of U-[13C17]-AFB1 5 ng/mL in acetonitrile and 20 μL of U-[13C17]-AFM1 10 ng/mL in acetonitrile were added to the sample. The diluted sample was centrifuged for 10 min at 3500 × g (RCF) before the injection of 10 μL into the UHPLC-HRMS system.

Immunoaffinity clean-up

Before analysis, all urine samples were equilibrated to room temperature and homogenized by shaking thoroughly. Aliquots of 2 mL urine were mixed with 10 mL of phosphate-buffered solution (PBS, pH = 7.4) and passed through the immunoaffinity column (IAC) for purification (Easy-extract® aflatoxins, from R-Biopharm, Darmstadt, Germany). The IAC was washed with 30 mL of H2O (10 + 10 + 10 mL), and then the toxins were eluted with 1 mL of MeOH (500 + 500 μL). Finally, 500 μL of eluted sample were added with 20 μL U-[13C17]-AFB1 2.5 ng/mL in ACN, 20 μL U-[13C17]-AFM1 5 ng/mL in ACN and 460 μL of H2O. A volume of 20 μL was injected into the UHPLC-HRMS system.

LC-HRMS analysis

Determination was performed by UHPLC-HRMS analysis. Chromatographic separation was performed using UHPLC Dionex UltiMate 3000® (Thermo Scientific, San Jose, CA, USA). An Acquity UPLC® BEH C18 column (1.7 μm, 100 × 2.1 mm, from Waters, Milford, MA, USA) was used at a temperature of 40 °C. The flow rate was 0.3 mL/min, and mobile phases A and B were respectively water and methanol containing 0.002% formic acid (v/v) and 2 mM mmol/L ammonium formate. The following gradient was applied: 20% B increase to 99% in 10 min, keep isocratic at 99% B for 4 min, from 14 to 14.6 min return to 20% B, and finally re-equilibrate the column at 20% B for 2.4 min. High-resolution MS analysis was performed using Q Exactive™ Quadrupole-Orbitrap™ equipped with heated electrospray ionization (HESI) source (Thermo Scientific, San Jose, CA, USA).
following ESI (+) parameters were used: source voltage 3.5 kV, in-source CID 18 eV, 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) for confirmatory purpose. Precursor ion, fragments and collision energy used for the determination of the selected mycotoxins are reported in Table 2. All analytical batches included analysis of appropriate extraction 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.

**Analytical quantification**

For mycotoxins quantification, an internal standard (ISTD) approach was adopted. The internal standard for AFB1 and AFM1 was the 13C isotope-labelled molecule in which all carbon atoms are substituted by the stable isotope 13C. Six points calibration curve was obtained by plotting the response ratio (standard area/13C area) versus the concentration expressed in pg/mL urine. The concentration ranges covered for dilute and shoot method were 5–100 pg/mL for AFB1 and 10–200 pg/mL for AFM1, corresponding to 50–1000 pg/mL urine and 100–2000 pg/mL urine for AFB1 and AFM1, respectively. For IAC method, the ranges were 5–50 pg/mL for AFB1 and 10–100 pg/mL for AFM1, corresponding to 2.5–25 pg/mL urine and 5–50 pg/mL urine for AFB1 and AFM1, respectively. The calibration curve was obtained by fitting the data with a linear regression model based on least squares method.

**Validation criteria**

Identification criteria were set for all the analysed mycotoxins. Linearity and limit of detection (LOD) and quantification (LOQ) of the analytical methods were assessed. Precision and trueness were assessed from repeated analyses on spiked blank urine 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 (RA). Extraction efficiency (RE) and matrix effect (SSE) were also evaluated for validation purpose.

**Method validation**

For both methods, linearity of the method was evaluated from six points calibration curves injected in triplicate for 3 consecutive days. 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.

According to the criteria reported in the SANTE/12089/2016 guidance document on identification of mycotoxins in food and feed (EC 2016), the retention time (RT) of the

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

| Volunteers       | Number of subjects | Mean age (range); years | Mean body weight (range); kg |
|------------------|--------------------|-------------------------|-----------------------------|
| Workers group    | 32                 | 53 (32–65)              | 80.1 (62–99)                |
| Controls group   | 29                 | 48 (33–63)              | 83.4 (64–125)               |
| Total            | 61                 | 53 (32–65)              | 80.1 (62–99)                |

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

| Compound          | Chemical formula | Precursor ion (m/z) [M + H]+ | Fragment (m/z) | NCEa |
|-------------------|------------------|------------------------------|----------------|-----|
| AFB1              | C17H12O6         | 313.07066                    | 285.07571; 241.04952 | 25  |
| 13C17-AFB1        | C17H12O6         | 330.12770                    | –              | –   |
| AFM1              | C17H12O7         | 329.06558; 351.04752b        | 273.07538; 229.04937 | 27  |
| 13C17-AFM1        | C17H12O6         | 346.12261; 368.10456b        | –              | –   |
| AFB1-N7-guanine   | C22H17N5O8       | 480.11499                    | 152.05678; 165.05499 | 40; 90 |

a Normalised collision energy

b [M + Na]+
analyte in the sample extract should correspond to that of the average of the calibration standards measured in the same sequence with a tolerance of ± 0.1 min. Moreover, for the ISTD added to the sample extract, the RT of the analyte should correspond to that of its labelled ISTD 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 one fragment that is specific for the selected analyte.

According to Wenzl et al. (2016), spiked blanks approach was used for LOD and LOQ assessment, by analysing the spiked sample 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. Calculations were carried out according to Eqs. 1 and 2.

\[
x_{LOD} = 3.9 \times \frac{s_{b1}}{b} \tag{1}
\]

\[
x_{LOQ} = 3.3 \times x_{LOD} \tag{2}
\]

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

For dilute and shoot method, the matrix effect was examined according to Sulyok et al. (2006) assessing the matrix-induced enhancement or suppression during analysis. For this purpose, calibration curves in solvent (5 calibration points in the range of 40–200 pg/mL for AFB\(_1\) and 80–400 pg/mL for AFM\(_1\), constructed by plotting signal intensity versus the analyte concentration) were compared with matrix-matched calibration curves (blank sample 1:10 diluted spiked at 5 levels, curves obtained by plotting the signal intensity against the actual spiking level). The slopes of the resulting calibration curves were used for signal suppression/enhancement (SSE) calculation (Eq. 3).

\[
SSE(\%) = 100 \times \frac{\text{slope (matrix-matched standard)}}{\text{slope (solvent standard)}} \tag{3}
\]

For IAC method, apparent recovery, matrix effect and extraction recovery were assessed on five different levels of contamination, including the calculated LOQ values, and for each level, triplicate analyses of spiked blank sample on 2 consecutive days were performed. The obtained data were used for apparent recovery (\(R_\text{A}\)), matrix effect and extraction recovery (\(R_\text{E}\)) calculations and for precision assessment. The \(R_\text{A}\) 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 (Eq. 4). In this case, the curves were obtained considering the area and not the ratio with the labelled internal standard added for each mycotoxin. The \(R_\text{A}\) 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. \(R_\text{A}\) was the parameter used for trueness evaluation.

\[
R_\text{A}(\%) = \frac{100 \times \text{slope (spiked sample)}}{\text{slope (solvent standard)}} \tag{4}
\]

The matrix effect was evaluated in terms of signal suppression/enhancement (SSE), and it was calculated, according to Eq. 5, as the ratio between the mean area of the labelled ISTD in the spiked sample extract and in the pure solvent standard solution.

\[
SSE(\%) = 100 \times \frac{\text{area U} - [13C_{17}] - AFB_1 \text{sample}}{\text{area U} - [13C_{17}] - AFB_1 \text{standard}} \tag{5}
\]

The \(R_\text{E}\), accounting to incomplete extraction of the analyte from the matrix, was calculated from \(R_\text{A}\) and SSE, according to Eq. 6.

\[
R_\text{E}(\%) = 100 \times \frac{R_\text{A}}{\text{SSE}} \tag{6}
\]

The effect of random errors on the measurements was assessed and quantified as the relative standard deviation (RSD) of repeated independent analyses conducted in intermediate conditions of repeatability within the laboratory (RSD\(_{LR}\)). Instrumental laboratory reproducibility of the LC-HRMS system was also evaluated by injecting on 3 consecutive days (inter-run) and in replicates (intra-run) a neat solvent standard solution (AFB\(_1\) 150 pg/mL and AFM\(_1\) 300 pg/mL). Moreover, the intermediate precision of the whole method was evaluated by analysing daily independent urine sample spiked at the same contamination level as an internal control sample (inter-day). The performance criteria for precision, quantified with standard deviation of repeatability within the laboratory (s\(_{LR}\)) and expressed in percentage as RSD\(_{LR}\), were set at 15% of variability, including any source of instrumental and analytical possible random errors. Precision was estimated in terms of intermediate precision RSD\(_{LR}\) of repeatability.

### Results and discussion

#### Sample preparation and LC-HRMS analysis

During method set-up, two urine:water dilution factors, namely, 1:5 and 1:10, were tested for dilute and shoot approach. The 1:10 dilution gave better results in terms of SSE and was selected for the analysis. The IAC purification step was introduced in order to reduce the LOD/LOQ values. The employed IAC contains specific antibodies to aflatoxins B\(_1\), B\(_2\), G\(_1\), G\(_2\)
and M₁, and no specific information was given by the supplier for AFB₁-N⁷-guanine. To evaluate the risk of adduct loss during purification, the synthetized adduct was applied to the IAC, eluted according to method protocol, and the presence of the adduct was confirmed by LC-HRMS identification.

HRMS conditions were set by direct infusion of standard solution for AFB₁ and AFM₁, while for AFB₁-N⁷-guanine, the selection of collision energy and specific fragments was guided by the work of Walton et al. (2001). AFB₁ produces sodiated adduct in a non-negligible amount when compared with protonated adduct during electrospray ionization step; although AFB₁ is unlikely to produce ammonium adduct, the presence of the ammonium in the mobile phase suppresses the sodiated adduct in favour of the protonated one. This is the reason for the presence in the mobile phases of formic acid and ammonium formate. For quantitative purpose, the protonated adducts were selected, for AFM₁, since it was not possible to reduce the sodiated adduct production by varying source parameters, the sum of the protonated and sodiated adduct was considered.

Validation parameters

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 R² > 0.990 was set as a criterion for calibration curve acceptability. In Table 3, the calibration curve ranges, the amount of ISTD added to each calibration level and the correlation coefficients (R²) obtained for each mycotoxin/matrix combination are reported.

The performance characteristics, in terms of Rₐ, SSE and Rₑ, are summarized in Table 4 together with LOD and LOQ values and the working range of the two analytical procedures. Both methods may be applied for quantitative analysis of AFB₁ and AFM₁ as well as for the evaluation of presence/absence of the AFB₁-N⁷-guanine adduct. The dilute and 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 and 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 RSDLR, were evaluated during dilute and shoot method validation. SSE percentages are very close to 100% due to the dilution applied to the urine sample; method precision was assessed by performing eight independent analyses at the LOQ level. The IAC clean-up method was fully validated, trueness was evaluated in terms of apparent recovery (Rₐ), while precision was assessed by laboratory reproducibility RSDLR measures. Although the IAC clean-up, which is a very selective approach, was used, the influence of the matrix was also evaluated, and the percentages of SSE for AFB₁ and AFM₁ found confirmed that the influence of the matrix on the instrumental response is very limited.

LOD and LOQ of analytical methods always represent a challenge being the bottleneck for the reliability of the analytical results and also for the further processing of the findings (i.e. data mining). Modern HRMS instruments make it possible to reach high sensitivities with low detection limits, and especially when methods are targeted, good benchmarks can be achieved. Among the most recent studies on the biomonitoring of aflatoxins, the lowest values for AFM₁ were found in the range of 0.13–0.6 pg/mL urine and in the 0.4–1.8 pg/mL urine for LOD and LOQ, respectively (Giolio et al. 2012; Romero et al. 2010). Although these values represent a gold standard benchmark, they are not covered by the strict performance requirements of accuracy, which instead were met at 10 pg/mL urine (Giolio et al. 2012) and 4 pg/mL urine (Romero et al. 2010). Thus, the LOD/LOQ values obtained in the IAC method, validated under strict performances, are in alignment with the findings in other biomonitoring works for AFM₁. Notwithstanding, all the positive samples were in the range of values between LOD and LOQ, revealing the crucial need to stress the method to reach lower levels. In conclusion, the general validation results obtained in this study are considered satisfactory either for screening or for confirmation, and the method is considered to suit for the production of accurate data for biomonitoring purposes.

Table 3  Calibration curve range, labelled internal standard concentration and correlation coefficients obtained for each mycotoxin/matrix combination are reported

| Method      | Calibration curve range (pg/mL) | Labelled standard (pg/mL) | R² (RSD, %)       |
|-------------|---------------------------------|---------------------------|-------------------|
|             | AFB₁                           | AFM₁                      |                   |
| Dilute and shoot | 5–100                          | 10–200                    |                   |
| IAC         | 2.5–50                          | 5–100                     |                   |
|             | U-[¹³C₁₇]-AFB₁                  | U-[¹³C₁₇]-AFM₁            |                   |
|             | 10                              | 20                        | 0.9965 (0.04)     |
|             | 50                              | 100                       | 0.9973 (0.09)     |
|             | AFB₁                           | AFM₁                      |                   |
|             | 0.9976 (0.20)                   | 0.9976 (0.10)             |                   |
Analytical results

Statistical analysis and data handling: left censored data

The hypothesis of normal distribution (Shapiro-Wilk test) was refused; thus non-parametrical tests were used for the statistical treatment of the analytical results. All possible differences between concentration levels of mycotoxins in exposed and nonexposed 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).

Under the rigid identification criteria for analyte determination, namely, the RT 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 data set as positive samples provided that the identification criteria were met. The results’ evaluation included also the reporting of the lower and upper bound (LB and UB) mean values (EFSA 2010). These values were calculated applying a substitution method for which in the LB calculations, the results lower than LOQ were substituted with zero, while in the UB, the results lower than LOQ were substituted with LOQ value depending on the method.

Analysis of samples

The collected urine samples were analysed first with the dilute and shoot method, through which none of the sample showed a measurable level of AFB\(_1\) or AFM\(_1\), including AFB\(_1\)-N\(_7\)-guanine which was not detected. To overcome the limitations coming from the detection limit threshold of the dilute and 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 IAC clean-up to clean and concentrate the urine sample. By using this method for reprocessing the urine samples, AFB\(_1\) and its adduct were not detected; AFM\(_1\), instead, was found in 14 samples (12%) within the range 1.9–10.5 pg/mL urine. Only one sample, coming from the workers’ group, showed a value above the LOQ (10.5 pg/mL urine), and it is a sample from the workers group. Tables 5 and 6 summarize the percentages of positive samples, maximum values found and mean values (LB-UB) for worker and control groups, respectively. It should be noted that when values reported for AFM\(_1\) are below the LOQ, they were considered as affected by a standard uncertainty higher than 25%, which was the performance criteria set for maximum standard uncertainty for the LOQ. The LB-UB values

| Table 4 | Performance characteristics obtained during validation for AFB\(_1\) and AFM\(_1\) in urine with dilute and shoot and IAC clean-up methods |
|---------|----------------------------------------------------------------------------------|
|         | Dilute and 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\(_{urine}\)) | 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 |

| Table 5 | Percentage of positive samples, maximum values found and mean values (LB-UB) for AFM\(_1\) in workers group samples |
|---------|----------------------------------------------------------------------------------|
|         | Positive\(^a\) (%) | Max\(^b\) (pg/mL\(_{urine}\)) | Mean (LB-UB) (pg/mL\(_{urine}\)) |
| Monday and Friday; subjects (\(n = 63\)) | 13 | 10.5 | 0.5–4.9 |
| Monday; subjects (\(n = 32\)) | 13 | 4.6\(^c\) | 0.4–4.48 |
| Friday; subjects (\(n = 31\)) | 13 | 10.5 | 0.6–5.0 |

\(^a\) Positive: values above LOD

\(^b\) Max: maximum value

\(^c\) Value below the LOQ
reflect the optimistic and pessimistic scenario range of possible mean values.

Figure 1 shows the data trend for AFM1 in urine for both groups, Monday and Friday sampling. On the left side, LB substitution method was applied, due to the high number of non-detected (87%), and box plot is flattened to zero. On the right side, the box plot reports all the positive values are reported. The band inside the box is the second quartile (P50, median). Dots indicate 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) can be found at the bottom and the top of the box, respectively.

No statistical difference for AFM1 was observed between Monday and Friday samples in each group (exposed and non-exposed workers). To note that among the positive results, two individuals of the exposed workers group showed AFM1 in both Monday and Friday deliveries (3.3 and 3.0 pg/mL urine and 4.6 and 10.5 pg/mL urine, Monday and Friday values for each individual, respectively). Further statistical analyses were performed merging data of Monday and Friday data (63 analyses for exposed workers group and 57 for non-exposed workers group). Eight samples (13%) resulted positive in the workers’ group where the highest contaminated sample was found (10.5 pg/mL urine); six samples (11%) were positive in the control group, and 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 2 days of urine delivery, no differences were highlighted.

The absence of AFB1 and its adduct, together with the absence of a statistical difference when the mean values of AFM1 for workers and control groups were compared, suggests that in this specific setting, no professional exposure occurs. Moreover, considering the very low level of AFM1 in the collected urine samples, the contribution from the diet to the overall exposure is to be considered negligible.

This study presents a method, performed by a high-resolution mass spectrometry (LC-HRMS) technique, to detect AFB1 and aflatoxin biomarkers, namely, AFM1 and aflatoxin-N7-guanine. If compared with the previous study conducted in 2014 (Ferri et al. 2017), the present work tackles with an improvement of method sensitivity (1.5 pg/mL urine versus 25 pg/mL urine in Ferri et al. (2017)).

### Table 6 Percentage of positive samples, maximum values found and mean values (LB-UB) for AFM1 in controls group samples

| Positive (%) | Max (pg/mL urine) | Mean (LB-UB) (pg/mL urine) |
|--------------|-------------------|-----------------------------|
| 11           | 4.1               | 0.3–4.8                     |
| 7            | 2.8c              | 0.2–4.8                     |
| 14           | 4.1c              | 0.4–4.7                     |

a Positive: values above LOD  
b Max: maximum value  
c Value below the LOQ

Fig. 1 Data trend for AFM1 in urine for exposed and control groups. Left side, mean LB 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).
High percentages of workers positive for AFB₁ were reported in several studies conducted in EU (Portugal, Italy, Denmark) (Olsen et al. 1988; Viegas et al. 2018; Ferri et al. 2017) emphasizing that occupational exposure might be critical in certain settings, such as feed plants, in which indoor areas can be conducive of highly contaminated dust particles. In this study, morning urine samples were collected during 1 working week from a cohort of occupationally exposed workers of a feed company and from a control group. The presence of only one positive sample of AFB₁ and the lack of statistical difference between mean values of AFM₁ in workers and control groups suggest that in this specific setting, the primary route of exposure to AFs is more likely to be attributed to the diet and not to the respiratory route when inhalation or dermal contact of aerosolized contaminated dusts occur. However, the attention and focus to AFs cannot be reduced to a no-risk situation; since, due to the direct correlation between aflatoxins occurrence and climate changes, a systematic monitoring of the health status of citizen (including workers) potentially exposed to dusts contaminated by these toxic compounds has to be duly undertaken.

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Compliance with ethical standard

The study has been approved by the Ethical Committee of Reggio Emilia Province and performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

Conflict of interest The authors declare that they have no conflict of interest.

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