

Mycotoxin Research

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

Manuscript Number:	MYRE-D-19-00053R2	
Full Title:	Optimization and validation of a LC-HRMS method for aflatoxins determination in urine samples	
Article Type:	Original Article	
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Funding Information:	EFSA (GP/EFSA/AFSCO/2017/05PERFORMAN CE)	MSc Carlo Brera
Abstract:	<p>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. Limit of detections were 0.8 and 1.5 pg/mL urine 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, aflatoxin M1, instead, was found in 14 samples (12%) within the range 1.9-10.5 pg/mL urine. 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 were compared suggests that in this specific setting, no professional exposure occurs. Furthermore, considering the very low level of aflatoxin</p>	

	M1 in the collected urine samples, also the contribution from the diet to the overall exposure is to be considered negligible.
Response to Reviewers:	<p>3RD REV (letter 19/12/2019) Dear reviewers, all requested changes has been accepted and text amended accordingly. see in detail</p> <ol style="list-style-type: none"> 1.Delete heading "Conclusions", this journal has no such section. Concluding remarks should be put directly after end of results and discussion. DONE 2.Do not use justified text, but type left-aligned throughout the manuscript without automated hyphenation (?). Then please check if some typos (extra space etc) become visible and correct im necessary. DONE 3.Move "Acknowledgements" section directly before the reference list section DONE 4.Temperature and other units: consistently add one space between number and unit, for example "2 °C". Only % should be placed directly at the number, for example "98.3%" DONE 5.Consistently use only SI units, for example L119 100 mmol/L phosphate, and look for other occurrences as well. DONE 6.L124 what does "Guanine dissolved in 0.1 N HCl (0.32 µmoles)" mean? a "mol" has no plural, just numbers. Replace N with mol/L DONE 7.Check reference list again carefully and correct/modify according to examples as given in the instructions for authors of this journal (for example, EU regulations, EC-regulations, FAO documents). Remove issue numbers in brackets, not necessary. At present, reference list is full of typos etc. NO capitals in reference text except first word. DONE 8.Figure legends should be placed on a separate page not directly at figures. There is a supplementary file in the first revision named capture list but this appears to be empty. Please place list of figure captions after the references on a separate page in the manuscript, before the tables. DONE 9.Tables: just 3 horizontal lines, one above and one below the main table body, and one separating the first parameter row from the data entries. No extra horizontal lines in the main table body. Tables 1 and 2 are ok. DONE



Mycotoxin Research Authorship and Disclosure Form

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

Article title (first few words)

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1 Optimization and validation of a LC-HRMS method for aflatoxins

2 determination in urine samples

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26 27 28 14 29 30 15 **Abstract**

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32 16 Mycotoxins exposure by inhalation and/or dermal contact can occur in different branches of industry especially where
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34 17 heavily dusty settings are present and the handling of dusty commodities is performed. This study aims to explore the
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36 18 possible contribution of the occupational exposure to aflatoxins by analysing urine samples for the presence of
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38 19 aflatoxins B₁ and M₁ and aflatoxin B₁-N⁷-Guanine adduct. The study was conducted in 2017 on two groups of
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50 25 adduct were not detected in the analysed samples, aflatoxin M₁, instead, was found in 14 samples (12%) within the
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56 28 that in this specific setting, no professional exposure occurs. Furthermore, considering the very low level of aflatoxin
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58 29 M₁ in the collected urine samples, also the contribution from the diet to the overall exposure is to be considered
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61 30 negligible.

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Keywords

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4 Biomonitoring, Biomarker, Mycotoxin, Aflatoxin, Metabolites, LC-Orbitrap, LC-HRMS

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Introduction

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Among xenobiotics, mycotoxins, secondary metabolites of fungal origin, are the most harmful hazards with high toxic

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potency and recognized adverse impacts on human and animal health. More than 500 mycotoxins are known, but

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scientific studies focus on those that exert carcinogenic and/or toxic activity, and only few of them are regulated

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worldwide (Stein et al. 2017; CAST 2003; FAO 2004). Among mycotoxins, aflatoxins (AFs) represent one of the most

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concerning class of chemical compounds with a focus of interest on aflatoxin B₁ (AFB₁) that, due to its acute and

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chronic toxic effects, have raised the interest of the scientific community. The primary target organ affected by aflatoxin

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B₁ exposure is the liver, and several epidemiological studies related AFB₁ exposure to cellular hepatocarcinoma, report

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it as one of the major cause of cancer-related deaths in different parts of the world (Wild and Turner 2002). AFB₁ is a

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genotoxic and carcinogenic substance, classified under group 1 by the International Agency for Research on Cancer

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(IARC 1993). AFs can occur in crops at pre-harvest, harvest and post-harvest stages as a result of different co-occurring

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environmental conditions and poor management practices (handling and storage). The expected global warming of +2

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°C is likely to cause a sensible climate change leading to conducive environmental conditions for AFs production in

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Northern-Europe, where currently no occurrence is significantly present (Battilani et al. 2016). Therefore, validating

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new methods for AFs determination becomes particularly relevant to be applied in newly exposed geographical regions.

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The most common route of exposure to mycotoxins is the ingestion through the diet due to the consumption of directly

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or indirectly contaminated food. Furthermore, humans and animals can also be exposed to mycotoxins through

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inhalation and/or dermal contact with contaminated dusts (Brera et al. 2002; Doi and Uetsuka 2014; Viegas et al. 2014,

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2017). Several studies reported a higher prevalence of lung carcinogenesis and bronchus and trachea tumours in

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workers exposed to aflatoxins contaminated dusts (McLaughlin et al. 1987; Olsen et al. 1988; Ghosh et al. 1997; Saad-

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Hussein et al. 2013, 2014), especially in branches of industry where the storage, loading, milling and handling of dusty

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commodities (such as grains, feed, spices, coffee, etc.) is performed. Due to their severe toxicological implications,

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exposure to aflatoxins must be characterized by an accurate evaluation. Commonly, two different approaches can be

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followed for targeting this issue: via dietary exposure assessment and/or via biomonitoring studies. The overall

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metabolic pathway of AFB₁ is quite complex and corresponds to the formation of a number of metabolites that could be

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61 associated not only to the dose of the parent mycotoxin, but also to the biological response to the exposure and to the
1
2 degree of individual sensitivity to adsorption and metabolism of the toxic agent (Groopman 1994). Validated exposure
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4 biomarkers for AFB₁ (urinary aflatoxin M₁, AFB₁-N⁷-Guanine) were established almost 20 years ago (Groopman et al.
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6 1993), they were critical in confirming aflatoxins as potent liver carcinogens, and more importantly, they are being used
7
8 to assess the effectiveness of intervention strategies (Cramer and Uetsuka 2017; Turner et al. 2012).

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10 Biomonitoring studies have been increased over the last 8 years. In a recent publication Viegas (Viegas et al. 2018)
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12 reviewed the use of biomonitoring in assessing occupational exposure to mycotoxin in different settings and 58% of the
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14 reviewed works assessed aflatoxins exposure. Despite the impossibility to distinguish between dietary and air-dust
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16 contamination, the literature review clearly showed that, under certain circumstances, workers were significantly more
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18 exposed than the control group (Malik et al. 2014; Saad-Hussein et al. 2014; Viegas et al. 2016). In Italy a first study on
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20 occupational exposure to aflatoxins was conducted in 2014 in two feed companies, to assess if workers occupied in
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22 dusty indoor settings were differently exposed than workers occupied in administrative units (control group) (Ferri et al.
23
24 2017). To monitor the situation and to assess the effect of new agricultural season, the same scheme of the study was
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26 replicated in 2017 within a different analytical framework, where also the guanine metabolite was included.

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

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38 Aflatoxins determination was performed by a high-resolution mass spectrometry (LC-HRMS) technique. For sample
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40 preparation, a dilute&shoot method and a quantitative method based on immunoaffinity column purification step were
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42 developed and fully validated. Moreover, due to the unavailability of commercial standard of AFB₁-N⁷-Guanine, the
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44 adduct was synthesized and used for the method set up and for qualitative analysis (presence/absence) in the collected
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46 samples.
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48 49 50 **Materials and Methods**

51 52 **Chemicals and reagents**

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54 Chemicals and solvents used for sample preparation were LC-MS grade. Methanol, formic acid and LC-MS grade
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56 water were purchased from Fisher Scientific (Milano, Italy), AFB₁ from *Aspergillus flavus* (purity ≥98%) was from
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58 Sigma-Aldrich (Darmstadt, Germany). The analytical reference standard of AFM₁ was purchased as stock solution (0.5
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60 µg/mL in acetonitrile) from Biopure® (Tulln, Austria). The isotopically labelled internal standards U-[¹³C₁₇]-AFB₁

92 (99.3% ¹³C) and U-[¹³C₁₇]-AFM₁ (98.3% ¹³C) were also purchased as acetonitrile solution (0.5 µg/mL) from Biopure®
93 (Tulln, Austria). The concentration reported in the certificate accompanying the reference standard purchased as
94 solution was considered for quantification purpose. The AFB₁ powder was reconstituted with 100% ACN and the
95 concentration was assessed by molar absorbance value following the procedure reported in the official Methods of
96 Analysis of AOAC (AOAC 2005). The AFB₁-N⁷-Guanine adduct was not commercially available at the moment of the
97 study and was synthesized as reported below.

98 *AFB₁-N⁷-Guanine adduct synthesis and identification*

99 The synthesis was conducted accordingly with Vidyasagar et al. (1997) as follows: meta-chloroperoxybenzoic acid
100 (MCPBA), 20 mg in 4 mL of dichloromethane, was washed with 100 mM mmol/L phosphate buffer, pH 7.4 (4 mL x 4).
101 The resulting MCPBA solution was passed through anhydrous sodium sulphate to remove residual water. AFB₁ (0.64
102 µmol) was dissolved in 250 µL of dichloromethane and was converted to AFB₁-8,9-epoxide by addition of 250 µL of
103 the above MCPBA solution (4 µmol) and 500 µL of 100 mM mmol/L phosphate buffer, pH 7.2. The reaction was
104 carried out at 5 °C for 100 min with continuous vigorous stirring. At the end of 100 min the buffer fraction was pipetted
105 out. 0.32 µmol of Guanine, previously dissolved in 0.1 mol/L HCl, were added to 500 µL of 100 mM mmol/L
106 phosphate buffer, pH 7.4 (maximum solubility of guanine in phosphate buffer was found to be 140 µg/mL). The buffer
107 with guanine was added to the tube containing AFB₁-8,9-epoxide in dichloromethane and the reaction was continued
108 for 60 min at 5 °C with continuous vigorous stirring. At the end of 60 min the reaction mixture was centrifuged at 4000
109 rpm for 5 min. The organic phase was separated and the buffer fraction was repeatedly washed with dichloromethane
110 (500 µL x 3 times). The adduct identification was based on the observation of the molecular ion and at least one
111 fragment specific for the analyte after injection in the LC-HRMS system, according to the guidance document on
112 identification of mycotoxins in food and feed (EC 2016). Due to the difficulties in assessing the concentration level of
113 the synthesized adduct, the diluted buffer fraction was used for testing the IAC cross reactivity during method
114 development and for a qualitative evaluation of presence/absence in the collected urine samples.

115 **Study design**

116 The investigation was conducted in the same feedstuff plant involved in the first study previously published by Ferri et
117 al. (2017). This second study was conducted within the framework of a larger project entitled “Biomonitoring data as a
118 tool for assessing aflatoxin B₁ exposure of workers – BIODAF” supported by EFSA (July 2017 - June 2018). The
119 project focused on aflatoxins and took into consideration urine and serum samples collection and analysis. Two

122 countries, Italy and Portugal, were involved in this study. The present paper reports the results obtained from the Italian
123 urine analyses.

124 Two groups of volunteers were enrolled, the “workers group”, corresponding to all workers in direct contact with some
125 risky activities such as the downloading of the raw material, its handling and the cleaning procedures; and the “control
126 group”, which included employees of the same company but designated to perform other activities considered not risky
127 for the absence of contaminated environmental dusts. The samples were collected on Monday and Friday morning in
128 one working week, Monday was chosen since it reflects a situation characterized by a preceding two-days washing
129 period and Friday was selected with the aim to verify a possible accumulation of AFs and consequent intake over the
130 week of sampling. The urine was collected in the morning and delivered to the medical staff before starting the morning
131 shift. A total of 61 male volunteers were enrolled (32 workers and 29 controls). The collected urine samples were stored
132 at -20 °C until analysis. The mean value and range for age and body weight of the enrolled volunteers are reported in
133 Table 1.

134 The study was conducted under the supervision of the Local Health Unit of Reggio Emilia and was approved by the
135 Ethical Committee of the Reggio Emilia province. All urine donors were informed about the purpose of the study and a
136 formal consent was individually signed prior to inclusion in the study.

138 **Sample preparation**

139 *Dilute&shoot sample preparation*

140 Before analysis, all urine samples were equilibrated to room temperature and homogenized by shaking thoroughly.

141 Aliquots of 100 µL urine were mixed with 860 µL of H₂O LC-MS grade; for quantification purpose 20 µL of U-[¹³C₁₇]-
142 AFB₁ 5 ng/mL in acetonitrile and 20 µL of U-[¹³C₁₇]-AFM₁ 10 ng/mL in acetonitrile were added to the sample. The
143 diluted sample was centrifuged for 10 minutes at 3500 x g (RCF) before the injection of 10 µL into the UHPLC-HRMS
144 system.

146 *Immunoaffinity clean-up*

147 Before analysis, all urine samples were equilibrated to room temperature and homogenized by shaking thoroughly.

148 Aliquots of 2 mL urine were mixed with 10 mL of phosphate buffered solution (PBS, pH=7.4) and passed through the
149 immunoaffinity column (IAC) for purification (Easy-extract[®] aflatoxins, from R-Biopharm, Darmstadt, Germany). The
150 IAC was washed with 30 mL of H₂O (10+10+10 mL), then the toxins were eluted with 1 mL of MeOH (500+500 µL).
151 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
152 ng/mL in ACN and 460 µL of H₂O. A volume of 20 µL was injected into the UHPLC-HRMS system.

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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, 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). The 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-MS²) 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&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

184 analyses on spiked blank urine samples. Precision was evaluated by calculating the intermediate relative standard
185 deviation (repeated analyses on different days), while trueness was estimated in terms of apparent recovery (R_A).
186 Extraction efficiency (R_E) and matrix effect (SSE) were also evaluated for validation purpose.

188 **Method validation**

189 For both methods, linearity of the method was evaluated from six points calibration curves injected in triplicate for three
190 consecutive days. Regression lines were plotted applying a linear regression model based on least squares method. The
191 linearity was assessed by visual checking of the residual plot of response ratios (plotted in y-direction) versus the
192 respective concentration levels (plotted in x-direction). The final estimated linearity model was verified using the lack-
193 of-fit test (significance of the test with p_{value} below 0.05), to confirm that the selected regression and linearity were
194 acceptable. Once visual checking of the residual and lack-of-fit test passed, the R squared coefficient was taken as a
195 measure of linearity.

196 According to the criteria reported in the SANTE/12089/2016 guidance document on identification of mycotoxins in
197 food and feed (EC 2016), the retention time (RT) of the analyte in the sample extract should correspond to that of the
198 average of the calibration standards measured in the same sequence with a tolerance of ± 0.1 min. Moreover, for the
199 ISTD added to the sample extract, the RT of the analyte should correspond to that of its labelled ISTD with a tolerance
200 of ± 0.05 min. For HRMS analysis identification is based on observation of the molecular ion (or, if not available,
201 adducts) and one fragment that is specific for the selected analyte.

202 According to Wenzl et al. (2016), spiked blanks approach was used for LOD and LOQ assessment, by analysing the
203 spiked sample in ten replicates under repeatability conditions. The variability expressed as standard deviation obtained
204 for the ten analyses of spiked blanks was used for the estimation of the critical value of LOD. Calculations were carried
205 out according to Equation 1 and 2.

$$207 \quad x_{LOD} = 3.9 \times \frac{s_{y,b}}{b} \quad (1)$$

$$208 \quad x_{LOQ} = 3.3 \times x_{LOD} \quad (2)$$

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

212 For dilute&shoot method, the matrix effect was examined according to Sulyok et al. (2006) assessing the matrix
213 induced enhancement or suppression during analysis. For this purpose, calibration curves in solvent (5 calibration points
214 in the range of 40-200 pg/mL for AFB₁ and 80-400 pg/mL for AFM₁, constructed by plotting signal intensity versus the

215 analyte concentration) were compared with matrix-matched calibration curves (blank sample 1:10 diluted spiked at 5
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216 levels, curves obtained by plotting the signal intensity against the actual spiking level). The slopes of the resulting
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217 calibration curves were used for signal suppression/enhancement (SSE) calculation (Equation 3).

$$218$$
$$219 \text{SSE}(\%) = 100 * \frac{\text{slope (matrix-matched standard)}}{\text{slope (solvent standard)}} \quad (3)$$

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For IAC method, apparent recovery, matrix effect and extraction recovery were assessed 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. The obtained data were used for apparent recovery (R_A), matrix effect and extraction recovery (R_E) calculations and for precision assessment. The R_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 (Equation 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_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.

$$331 \text{R}_A(\%) = 100 * \frac{\text{slope (spiked sample)}}{\text{slope (solvent standard)}} \quad (4)$$

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

$$437 \text{SSE}(\%) = 100 \times \frac{\text{area } U-[13C_{17}]-AFB_1 \text{ sample}}{\text{area } U-[13C_{17}]-AFB_1 \text{ standard}} \quad (5)$$

The R_E , accounting to incomplete extraction of the analyte from the matrix, was calculated from R_A and SSE, according to Equation 6.

$$542 \text{R}_E(\%) = 100 * \frac{\text{R}_A}{\text{SSE}} \quad (6)$$

244 The effect of random errors on the measurements were assessed and quantified as the relative standard deviation (RSD)
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245 of repeated independent analyses conducted in intermediate conditions of repeatability within the laboratory (RSD_{LR}).
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246 Instrumental laboratory reproducibility of the LC-HRMS system was also evaluated by injecting on three consecutive
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247 days (inter-run) and in replicates (intra-run) a neat solvent standard solution (AFB_1 150 pg/mL and AFM_1 300 pg/mL).
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248 Moreover, the intermediate precision of the whole method was evaluated by analyzing daily independent urine sample
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249 spiked at the same contamination level as an internal control sample (inter-day). The performance criteria for precision,
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250 quantified with standard deviation of repeatability within the laboratory (S_{LR}) and expressed in percentage as RSD_{LR} ,
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251 was set at 15% of variability, including any source of instrumental and analytical possible random errors. Precision was
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252 estimated in terms of intermediate precision RSD_{LR} of repeatability.
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254 **Results and Discussion**

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255 **Sample preparation and LC-HRMS analysis**

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256 During method set up two urine:water dilution factors, namely 1:5 and 1:10, were tested for dilute&shoot approach. The
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257 1:10 dilution gave better results in terms of SSE and was selected for the analysis. The IAC purification step was
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258 introduced in order to reduce the LOD/LOQ values. The employed IAC contains specific antibodies to aflatoxins B_1 ,
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259 B_2 , G_1 , G_2 and M_1 , no specific information was given by the supplier for AFB_1-N^7 -Guanine. To evaluate the risk of
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260 adduct loss during purification, the synthesized adduct was applied to the IAC, eluted according to method protocol and
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261 the presence of the adduct was confirmed by LC-HRMS identification.

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262 HRMS conditions were set by direct infusion of standard solution for AFB_1 and AFM_1 , while for AFB_1-N^7 -Guanine the
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263 selection of collision energy and specific fragments were guided by the work of Walton et al. (2001). AFB_1 produces
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264 sodiated adduct in a non negligible amount when compared with protonated adduct during electrospray ionization step,
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265 although AFB_1 is unlikely produce ammonium adduct, the presence of the ammonium in the mobile phase suppresses
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266 the sodiated adduct in favour of the protonated one. This is the reason for the presence in the mobile phases of formic
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267 acid and ammonium formate. For quantitative purpose the protonated adducts were selected, for AFM_1 , since it was not
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268 possible to reduce the sodiated adduct production by varying source parameters, the sum of the protonated and sodiated
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269 adduct was considered.
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52 53 54 55 **Validation parameters**

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5772 Linearity was checked in the working range by the lack-of-fit test based on the analysis of variance (F test with p value
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5773 <0.05) and the plot of the residual values randomly distributed around zero, confirming the linearity. During routine
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5774 analytical sessions an $R^2 > 0.990$ was set as a criterion for calibration curve acceptability. In Table 3 the calibration
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275 curve ranges, the amount of ISTD added to each calibration level and the correlation coefficients (R^2) obtained for each
276 mycotoxin/matrix combination are reported.

277 The performance characteristics, in terms of R_A , SSE and R_E , are summarized in Table 4 together with LOD and LOQ
278 values and the working range of the two analytical procedures. Both methods may be applied for quantitative analysis
279 of AFB₁ and AFM₁ as well as for the evaluation of presence/absence of the AFB₁-N⁷-Guanine adduct. The dilute&shoot
280 method is characterized by higher LOD and LOQ values when compared with the IAC clean-up method, but on the
281 other hand the dilute&shoot approach is very quick and characterized by a conservative approach with respect to the
282 sample, giving the possibility of a retrospective analysis on the acquired data. Due to the absence of a sample pre-
283 treatment only matrix effect, in terms of SSE, and precision, in terms of RSD_{LR} , were evaluated during dilute&shoot
284 method validation. SSE percentages are very close to 100% due to the dilution applied to the urine sample; method
285 precision was assessed by performing 8 independent analyses at the LOQ level. The IAC clean-up method was fully
286 validated, trueness was evaluated in terms of apparent recovery (R_A) while precision was assessed by laboratory
287 reproducibility RSD_{LR} measures. Although the IAC clean-up, which is a very selective approach, was used, the
288 influence of the matrix was also evaluated, and the percentages of SSE for AFB₁ and AFM₁ found confirmed that the
289 influence of the matrix on the instrumental response is very limited.

290 LOD and LOQ of analytical methods always represent a challenge being the bottleneck for the reliability of the
291 analytical results and also for the further processing of the findings (i.e. data mining). Modern HRMS instruments
292 makes it possible to reach high sensitivities with low detection limits, and especially when methods are targeted, good
293 benchmarks can be achieved. Among the most recent studies on the biomonitoring of aflatoxins, the lowest values for
294 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
295 (Giolo et al. 2012; Romero et al. 2010). Although these values represent a gold standard benchmark, they are not
296 covered by the strict performance requirements of accuracy, which instead were met at 10 pg/mL_{urine} (Giolo et al. 2012)
297 and 4 pg/mL_{urine} (Romero et al. 2010). Thus, the LOD/LOQ values obtained in the IAC method, validated under strict
298 performances, are in alignment with the findings in other biomonitoring works for AFM₁. Notwithstanding, all the
299 positive samples were in the range of values between LOD and LOQ, revealing the crucial need to stress the method to
300 reach lower levels. In conclusion, the general validation results obtained in this study are considered satisfactory either
301 for screening and for *confirmation* and the method is considered to suit for the production accurate data for
302 biomonitoring purposes.

304 **Analytical results**

305 *Statistical analysis and Data handling - Left censored data*

306 The hypothesis of normal distribution (Shapiro–Wilk test) was refused, thus non-parametrical tests were used for the
307 statistical treatment of the analytical results. All possible differences between concentration levels of mycotoxins in
308 exposed and non-exposed groups were explored by a Wilcoxon rank-sum test. To assess the correlation between
309 mycotoxin levels, a Spearman’s rank correlation coefficient (or Spearman’s rho) was used. All tests were conducted
310 with a level of significance of 5%. Analyses were conducted by means of STATA14 software (Stata/IC 14.0, Copyright
311 1985–2015 StataCorp LP). Under the rigid identification criteria for analyte determination, namely the RT criteria
312 (RT±0.1 min with respect to the standard RT) and the presence of the precursor ion and at least one characteristic
313 fragment for each considered analyte, it was decided to include and report also all the values below LOQ obtained by
314 the interpolation of the calibration curve. Thus, values lower than LOQ were reported in the dataset as positive samples
315 provided that the identification criteria were met. The results evaluation included also the reporting of the lower and
316 upper bound (LB and UB) mean values (EFSA 2010). These values were calculated applying a substitution method for
317 which in the LB calculations the results lower than LOQ were substituted with zero, while in the UB the results lower
318 than LOQ were substituted with LOQ value depending on the method.

320 *Analysis of samples*

321 The collected urine samples were analysed first with the dilute&shoot method, through which none of the sample
322 showed a measurable level of AFB₁ or AFM₁, including AFB₁-N⁷-Guanine which was not detected. To overcome the
323 limitations coming from the detection limit threshold of the dilute&shoot method and verify that the negativity of the
324 results could be caused by the level of LOD/LOQ declared, it was decided to set up and validate a method with lower
325 LOQ. A purification step was introduced using an IAC clean-up to clean and concentrate the urine sample. By using
326 this method for reprocessing the urine samples, AFB₁ and its adduct were not detected, AFM₁, instead, was found in 14
327 samples (12%) within the range 1.9-10.5 pg/mL_{urine}. Only one sample, coming from the workers’ group, showed a value
328 above the LOQ (10.5 pg/mL_{urine}) and it is a sample from the workers group. Tables 5 and 6 summarize the percentages
329 of positive samples, maximum values found, and mean values (LB-UB) for worker and control groups, respectively. It
330 should be noted that when values reported for AFM₁ are below the LOQ they were considered as affected by a standard
331 uncertainty higher than 25%, which was the performance criteria set for maximum standard uncertainty for the LOQ.
332 The LB-UB values reflect the optimistic and pessimistic scenario range of possible mean values.

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

336 median). Dots indicates suspected outliers. Whiskers are set from minimum to maximum value. First and third quartiles
337 (P25 and P75) can be found at the bottom and the top of the box, respectively.
338 No statistical difference for AFM₁ was observed between Monday and Friday samples in each group (exposed and non-
339 exposed workers). To note that among the positive results two individuals of the exposed workers group showed AFM₁
340 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
341 each individual, respectively. Further statistical analyses were performed merging data of Monday and Friday data (63
342 analyses for exposed workers group and 57 for non-exposed workers group). Eight samples (13%) resulted positive in
343 the workers' group where the highest contaminated sample was found (10.5 pg/mL_{urine}); six samples (11%) were
344 positive in the control group, the higher detected value was 4.1 pg/mL_{urine}. In order to find differences among the
345 positive values found in workers and control group, a Wilcoxon rank-sum test was performed but no statistical
346 significances were highlighted; even exploring the two days of urine delivery, no differences were highlighted.
347 The absence of AFB₁ and its adduct, together with the absence of a statistical difference when the mean values of AFM₁
348 for workers and control groups were compared, suggests that in this specific setting, no professional exposure occurs.
349 Moreover, considering the very low level of AFM₁ in the collected urine samples, also the contribution from the diet to
350 the overall exposure is to be considered negligible.

351 This study presents a method, performed by a high-resolution mass spectrometry (LC-HRMS) technique, to detect
352 AFB₁ and aflatoxin biomarkers, namely AFM₁ and aflatoxin-N7-Guanine. If compared with the previous study
353 conducted in 2014 (Ferri et al. 2017), the present work tackles with an improvement of method sensitivity (1.5
354 pg/mL_{urine} versus 25 pg/mL_{urine} in Ferri et al. (2017). High percentages of workers positive for AFB₁ were reported in
355 several studies conducted in EU (Portugal, Italy, Denmark) (Olsen et al. 1988; Viegas et al. 2018; Ferri et al. 2017)
356 emphasizing that occupational exposure might be critical in certain settings, such as feed plants, in which indoor areas
357 can be conducive of highly contaminated dust particles. In this study, morning urine samples were collected during one
358 working week from a cohort of occupational exposed workers of a feed company and from a control group. The
359 presence of only one positive sample of AFB₁ and the lack of statistical difference between mean values of AFM₁ in
360 workers and control groups, suggests that in this specific setting, the primary route of exposure to AFs is more likely to
361 be attributed to the diet and not to the respiratory route when inhalation or dermal contact of aerosolized contaminated
362 dusts occur. However, the attention and focus to AFs can't never be reduced to a no-risk situation, since, due to the
363 direct correlation between aflatoxins occurrence and climate changes, a systematic monitoring of the health status of
364 citizen (including workers) potentially exposed to dusts contaminated by these toxic compounds, has to be duly
365 undertaken.

367 **Compliance with ethical standard**

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

371 **Conflict of Interest** The authors declare no conflict of interest.

373 **Acknowledgements**

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

378 **Gabriele Moracci, Paola De Santis, Maria Cristina Barea Toscan and Giuliana Verrone (Istituto Superiore di
379 Sanità), and Massimo Magnani (Servizio Prevenzione Sicurezza Ambienti di Lavoro) are acknowledged for their
380 technical assistance.**

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486 **Figure 1** Data trend for AFM1 in urine for exposed and control groups. Left side, mean LB values; right side, mean
487 positive values. The horizontal band (inside the box) is the second quartile (P50, median). Dots are suspected outliers.
488 Whiskers are set from minimum to maximum value. The bottom and the top of the box are the first and third quartiles
489 (P25 and P75).

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517 **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	-	-

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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)	NCE ^a
AFB ₁	C ₁₇ H ₁₂ O ₆	313.07066	285.07571; 241.04952	25
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
¹³ C ₁₇ -AFM ₁	C ₁₇ H ₁₂ O ₆	346.12261 + 368.10456 ^b	-	-
AFB ₁ -N ⁷ -Guanine	C ₂₂ H ₁₇ N ₅ O ₈	480.11499	152.05678, 165.05499	40; 90

^aNormalised Collision Energy; ^b[M+Na]⁺

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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)		R ² (RSD, %)	
	AFB ₁	AFM ₁	U-[¹³ C ₁₇]-AFB ₁	U-[¹³ C ₁₇]-AFM ₁	AFB ₁	AFM ₁
Dilute&shoot	5–100	10–200	10	20	0.9965 (0.04)	0.9967 (0.20)
IAC	2.5–50	5–100	50	100	0.9973 (0.09)	0.9976 (0.10)

598 **Table 4** Performance characteristics obtained during validation for AFB₁ and AFM₁ in urine with
 599 dilute&shoot and IAC clean-up methods
 600

	Dilute&shoot method		IAC clean-up method	
	AFB ₁	AFM ₁	AFB ₁	AFM ₁
601 LOD (pg/mL_{urine})	20	40	0.8	1.5
602 LOQ (pg/mL_{urine})	50	100	2.5	5.0
603 Working range (pg/mL_{urine})	50.0–1000.0	100.0–2000.0	2.5–25.0	5.0–50.0
604 R_A (%)	-	-	101	98
605 R_E (%)	-	-	97	92
606 SSE (%)	82	111	104	107
607 RSD_r (%)	8	11	6	12

622 **Table 5.** Percentage of positive samples, maximum values found and mean values (LB-UB) for AFM₁ in workers group
 623 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

624 ^aPositive: values above LOD

625 ^bMax: maximum value

626 ^cvalue below the LOQ

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

	Positive ^a (%)	Max ^b (pg/mL _{urine})	Mean (LB-UB) (pg/mL _{urine})
Monday and Friday; subjects (n=57)	11	4.1	0.3-4.8
Monday; subjects (n=29)	7	2.8 ^c	0.2-4.8
Friday; subjects (n=28)	14	4.1 ^c	0.4-4.7

653 ^aPositive: values above LOD

654 ^bMax: maximum value

655 ^cvalue below the LOQ

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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. Limit of detections were 0.8 and 1.5 pg/mL_{urine} 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, aflatoxin M₁, instead, was found in 14 samples (12%) within the range 1.9-10.5 pg/mL_{urine}. 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 were compared suggests

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31 that in this specific setting, no professional exposure occurs. Furthermore, considering the very low level of aflatoxin
32 M₁ in the collected urine samples, also the contribution from the diet to the overall exposure is to be considered
10 negligible.
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35 **Keywords**
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35 Biomonitoring, Biomarker, Mycotoxin, Aflatoxin, Metabolites, LC-Orbitrap, LC-HRMS
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20 **Introduction**

21 Among xenobiotics, mycotoxins, secondary metabolites of fungal origin, are the most harmful hazards with high toxic
22 potency and recognized adverse impacts on human and animal health. More than 500 mycotoxins are known, but
23 scientific studies focus on those that exert carcinogenic and/or toxic activity, and only few of them are regulated
24 worldwide (Stein et al. 2017; CAST 2003; FAO 2004). Among mycotoxins, aflatoxins (AFs) represent one of the most
25 concerning class of chemical compounds with a focus of interest on aflatoxin B₁ (AFB₁) that, due to its acute and
27 chronic toxic effects, have raised the interest of the scientific community. The primary target organ affected by aflatoxin
28 B₁ exposure is the liver, and several epidemiological studies related AFB₁ exposure to cellular hepatocarcinoma, report
30 it as one of the major cause of cancer-related deaths in different parts of the world (Wild ~~et al~~ and Turner- 2002). AFB₁
31 is a genotoxic and carcinogenic substance, classified under group 1 by the International Agency for Research on Cancer
32 (IARC 1993). AFs can occur in crops at pre-harvest, harvest and post-harvest stages as a result of different co-occurring
33 environmental conditions and poor management practices (handling and storage). The expected global warming of +2
34 °C is likely to cause a sensible climate change leading to conducive environmental conditions for AFs production in
35 Northern-Europe, where currently no occurrence is significantly present (Battilani et al. 2016). Therefore, validating
36 new methods for AFs determination becomes particularly relevant to be applied in newly exposed geographical regions.
37 The most common route of exposure to mycotoxins is the ingestion through the diet due to the consumption of directly
38 or indirectly contaminated food. Furthermore, humans and animals can also be exposed to mycotoxins through
39 inhalation and/or dermal contact with contaminated dusts (Brera et al. 2002; Doi ~~and Uetsuka et al.~~ 2014; Viegas et al.
40 2014,~~;~~ Viegas et al. 2017). Several studies reported a higher prevalence of lung carcinogenesis and bronchus and
41 trachea tumours in workers exposed to aflatoxins contaminated dusts (McLaughlin et al. 1987; Olsen et al. 1988; Ghosh
42 et al. 1997; Saad-Hussein et al. 2013,~~;~~ Saad-Hussein et al. 2014), especially in branches of industry where the storage,
43 loading, milling and handling of dusty commodities (such as grains, feed, spices, coffee, etc.) is performed. Due to their
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8 severe toxicological implications, exposure to aflatoxins must be characterized by an accurate evaluation. Commonly,
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10 two different approaches can be followed for targeting this issue: via dietary exposure assessment and/or via
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12 biomonitoring studies. The overall metabolic pathway of AFB₁ is quite complex and corresponds to the formation of a
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14 number of metabolites that could be associated not only to the dose of the parent mycotoxin, but also to the biological
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16 response to the exposure -and to the degree of individual sensitivity to adsorption and metabolism of the toxic agent
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18 (Groopman 1994). Validated exposure biomarkers for AFB₁ (urinary aflatoxin M₁, AFB₁-N⁷-Guanine) were established
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20 almost 20 years ago (Groopman et al. 1993), they were critical in confirming aflatoxins as potent liver carcinogens, and
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22 more importantly, they are being used to assess the effectiveness of intervention strategies (Cramer ~~et al.~~ and Uetsuka
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24 2017; Turner et al. 2012).

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

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

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

63 **Materials and Methods**

64 **Chemicals and reagents**

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92 Chemicals and solvents used for sample preparation were LC-MS grade. Methanol, formic acid and LC-MS grade
93 water were purchased from Fisher Scientific (Milano, Italy), AFB₁ from *Aspergillus flavus* (purity ≥98%) was from
10 Sigma-Aldrich (Darmstadt, Germany). The analytical reference standard of AFM₁ was purchased as stock solution (0.5
94 µg/mL in acetonitrile) from Biopure® (Tulln, Austria). The isotopically labelled internal standards U-[¹³C₁₇]-AFB₁
101 (99.3% ¹³C) and U-[¹³C₁₇]-AFM₁ (98.3% ¹³C) were also purchased as acetonitrile solution (0.5 µg/mL) from Biopure®
102 (Tulln, Austria). The concentration reported in the certificate accompanying the reference standard purchased as
16 solution was considered for quantification purpose. The AFB₁ powder was reconstituted with 100% ACN and the
98 concentration was assessed by molar absorbance value following the procedure reported in the official Methods of
19 Analysis of AOAC (AOAC 2005). The AFB₁-N⁷-Guanine adduct was not commercially available at the moment of the
20 study and was synthesized as reported below.

104 *AFB₁-N⁷-Guanine adduct synthesis and identification*

25 The synthesis was conducted accordingly with Vidyasagar et al. (1997) as follows: meta-chloroperoxybenzoic acid
104 (MCPBA), 20 mg in 4 mL of dichloromethane, was washed with 100 mM mmol/L phosphate buffer, pH 7.4 (4 mL x 4).
107 The resulting MCPBA solution was passed through anhydrous sodium sulphate to remove residual water. AFB₁ (0.64
108 µmoles) was dissolved in 250 µL of dichloromethane and was converted to AFB₁-8,9-epoxide by addition of 250 µL of
109 the above MCPBA solution (4 µmoles) and 500 µL of 100 mM mmol/L phosphate buffer, pH 7.2. The reaction was
110 carried out at 5 °C for 100 min with continuous vigorous stirring. At the end of 100 min the buffer fraction was pipetted
111 out. 0.32 µmoles of Guanine, previously dissolved in 0.1 mol/L N HCl, (0.32 µmoles) was were added to taken in 500
112 µL of 100 mM mmol/L phosphate buffer, pH 7.4 (maximum solubility of guanine in phosphate buffer was found to be
113 140 µg/mL). The buffer with guanine was added to the tube containing AFB₁-8,9-epoxide in dichloromethane and the
114 reaction was continued for 60 min at 5 °C with continuous vigorous stirring. At the end of 60 min the reaction mixture
115 was centrifuged at 4000 rpm for 5 min. The organic phase was separated and the buffer fraction was repeatedly washed
116 with dichloromethane (500 µL x 3 times). The adduct identification was based on the observation of the molecular ion
117 and at least one fragment specific for the analyte after injection in the LC-HRMS system, according to the guidance
118 document on identification of mycotoxins in food and feed (European EC Commission 2016). Due to the difficulties in
119 assessing the concentration level of the synthesized adduct, the diluted buffer fraction was used for testing the IAC cross
120 reactivity during method development and for a qualitative evaluation of presence/absence in the collected urine
121 samples.

123 **Study design**

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123 The investigation was conducted in the same feedstuff plant involved in the first study previously published by Ferri et
124 al. (2017). This second study was conducted within the framework of a larger project entitled “Biomonitoring data as a
125 tool for assessing aflatoxin B₁ exposure of workers – BIODAF” supported by EFSA (July 2017 - June 2018). The
126 project focused on aflatoxins and took into consideration urine and serum samples collection and analysis. Two
127 countries, Italy and Portugal, were involved in this study. The present paper reports the results obtained from the Italian
128 urine analyses.
129 Two groups of volunteers were enrolled, the “workers group”, corresponding to all workers in direct contact with some
130 risky activities such as the downloading of the raw material, its handling and the cleaning procedures; and the “control
131 group”, which included employees of the same company but designated to perform other activities considered not risky
132 for the absence of contaminated environmental dusts. The samples were collected on Monday and Friday morning in
133 one working week, Monday was chosen since it reflects a situation characterized by a preceding two-days washing
134 period and Friday was selected with the aim to verify a possible accumulation of AFs and consequent intake over the
135 week of sampling. The urine was collected in the morning and delivered to the medical staff before starting the morning
136 shift. A total of 61 male volunteers were enrolled (32 workers and 29 controls). The collected urine samples were stored
137 at -20 °C until analysis. The mean value and range for age and body weight of the enrolled volunteers are reported in
138 Table 1.
139 The study was conducted under the supervision of the Local Health Unit of Reggio Emilia and was approved by the
140 Ethical Committee of the Reggio Emilia province. All urine donors were informed about the purpose of the study and a
141 formal consent was individually signed prior to inclusion in the study.
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143 **Sample preparation**

144 *Dilute & shoot sample preparation*

145 Before analysis, all urine samples were equilibrated to room temperature and homogenized by shaking thoroughly.
146 Aliquots of 100 µL urine were mixed with 860 µL of H₂O LC-MS grade; for quantification purpose 20 µL of U-[¹³C₁₇]-
147 AFB₁ 5 ng/mL in acetonitrile and 20 µL of U-[¹³C₁₇]-AFM₁ 10 ng/mL in acetonitrile were added to the sample. The
148 diluted sample was centrifuged for 10 minutes at 3500 x g (RCF) before the injection of 10 µL into the UHPLC-HRMS
149 system.
150

150 *Immunoaffinity clean-up*

151 Before analysis, all urine samples were equilibrated to room temperature and homogenized by shaking thoroughly.
152 Aliquots of 2 mL urine were mixed with 10 mL of phosphate buffered solution (PBS, pH=7.4) and passed through the
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154 immunoaffinity column (IAC) for purification (Easy-extract® aflatoxins, from R-Biopharm, Darmstadt, Germany). The
155 IAC was washed with 30 mL of H₂O (10+10+10 mL), then the toxins were eluted with 1 mL of MeOH (500+500 µL).
156 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
157 ng/mL in ACN and 460 µL of H₂O. A volume of 20 µL was injected into the UHPLC-HRMS system.

158 159 160 161 162 163 164 165 **LC-HRMS Analysis**

166 Determination was performed by UHPLC-HRMS analysis. Chromatographic separation was performed using UHPLC
167 Dionex UltiMate 3000® (Thermo Scientific, San Jose, CA, USA). An Acquity UPLC® BEH C18 column (1.7 µm, 100
168 × 2.1 mm, from Waters, Milford, MA, USA) was used at a temperature of 40 °C. The flow rate was 0.3 mL/min,
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170 mobile phases A and B were respectively water and methanol containing 0.002% formic acid (v/v) and 2 mM mmol/L
171 ammonium formate. The following gradient was applied: 20% B increase to 99% in 10 min, keep isocratic at 99% B for
172 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
173 MS analysis was performed using Q Exactive™ Quadrupole-Orbitrap™ equipped with Heated ElectroSpray Ionization
174 (HESI) source (Thermo Scientific, San Jose, CA, USA). The following ESI (+) parameters were used: source voltage
175 3.5 kV, in-source CID 18 eV, capillary temperature 320 °C, auxiliary gas heater temperature 350 °C, sheath gas flow
176 40, S-lens RF level 75 and auxiliary gas flow 14. The MS acquisition was performed in Full Scan/Data Dependent (full
177 MS/dd-MS²) for confirmatory purpose. Precursor ion, fragments and collision energy used for the determination of the
178 selected mycotoxins are reported in Table 2. All analytical batches included analysis of appropriate extraction and
179 solvent blanks, solvent calibration curves at the beginning and end of the analytical batch, and injection of a calibration
180 level every 10 sample injections to ensure LC-MS stability throughout the run. For data acquisition and processing,
181 Xcalibur™ software 4.0.27.19 was used.

182 183 184 185 186 187 188 189 190 191 **Analytical quantification**

192 For mycotoxins quantification an internal standard (ISTD) approach was adopted. The internal standard for AFB₁ and
193 AFM₁ was the ¹³C isotope labelled molecule in which all carbon atoms are substituted by the stable isotope ¹³C. Six
194 points calibration curve was obtained by plotting the response ratio (standard area/¹³C area) versus the concentration
195 expressed in pg/mL_{urine}. The concentration ranges covered for dilute&shoot method were 5-100 pg/mL for AFB₁ and
196 10-200 pg/mL for AFM₁, corresponding to 50-1000 pg/mL_{urine} and 100-2000 pg/mL_{urine} for AFB₁ and AFM₁,
197 respectively. For IAC method the ranges were 5-50 pg/mL for AFB₁ and 10-100 pg/mL for AFM₁, corresponding to
198 2.5-25 pg/mL_{urine} and 5-50 pg/mL_{urine} for AFB₁ and AFM₁, respectively. The calibration curve was obtained by fitting
199 the data with a linear regression model based on least squares method.

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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 (R_A). Extraction efficiency (R_E) 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 three 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 (European Commission 2016), the retention time (RT) of the 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 Equation 1 and 2.

$$x_{LOD} = 3.9 \times \frac{s_{y,b}}{b} \quad (1)$$

$$x_{LOQ} = 3.3 \times x_{LOD} \quad (2)$$

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

217 For dilute&shoot method, the matrix effect was examined according to Sulyok et al. (2006) assessing the matrix
218 induced enhancement or suppression during analysis. For this purpose, calibration curves in solvent (5 calibration points
219 in the range of 40-200 pg/mL for AFB₁ and 80-400 pg/mL for AFM₁, constructed by plotting signal intensity versus the
220 analyte concentration) were compared with matrix-matched calibration curves (blank sample 1:10 diluted spiked at 5
221 levels, curves obtained by plotting the signal intensity against the actual spiking level). The slopes of the resulting
222 calibration curves were used for signal suppression/enhancement (SSE) calculation (Equation 3).

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$$SSE(\%) = 100 * \frac{\text{slope (matrix-matched standard)}}{\text{slope (solvent standard)}} \quad (3)$$

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226 For IAC method, apparent recovery, matrix effect and extraction recovery were assessed on 5 different levels of
227 contamination, including the calculated LOQ values, for each level triplicate analyses of spiked blank sample on 2
228 consecutive days were performed. The obtained data were used for apparent recovery (R_A), matrix effect and extraction
229 recovery (R_E) calculations and for precision assessment. The R_A is calculated as the ratio between the slope of the
230 spiked sample curve, obtained from the spiked samples, and the slope of the calibration curve in pure solvent (Equation
231 4). In this case, the curves were obtained considering the area and not the ratio with the labelled internal standard added
232 for each mycotoxin. The R_A represents the influence of the whole analytical process (sample preparation +
233 determination) on the signal and it is also referred to as overall or total recovery of a method. R_A was the parameter
234 used for trueness evaluation.

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$$R_A(\%) = 100 * \frac{\text{slope (spiked sample)}}{\text{slope (solvent standard)}} \quad (4)$$

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242 The matrix effect was evaluated in terms of Signal Suppression/Enhancement (SSE) and it was calculated, according to
243 Equation 5, as the ratio between the mean area of the labelled ISTD in the spiked sample extract and in the pure solvent
244 standard solution.

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

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244 The R_E , accounting to incomplete extraction of the analyte from the matrix, was calculated from R_A and SSE, according
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249 to Equation 6.

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$$R_E(\%) = 100 * \frac{R_A}{SSE} \quad (6)$$

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15 The effect of random errors on the measurements were assessed and quantified as the relative standard deviation (RSD)
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16 of repeated independent analyses conducted in intermediate conditions of repeatability within the laboratory (RSD_{LR}).
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18 Instrumental laboratory reproducibility of the LC-HRMS system was also evaluated by injecting on three consecutive
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19 days (inter-run) and in replicates (intra-run) a neat solvent standard solution (AFB₁ 150 pg/mL and AFM₁ 300 pg/mL).
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21 Moreover, the intermediate precision of the whole method was evaluated by analyzing daily independent urine sample
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22 spiked at the same contamination level as an internal control sample (inter-day). The performance criteria for precision,
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23 quantified with standard deviation of repeatability within the laboratory (S_{LR}) and expressed in percentage as RSD_{LR} ,
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25 was set at 15% of variability, including any source of instrumental and analytical possible random errors. Precision was
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27 estimated in terms of intermediate precision RSD_{LR} of repeatability.
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259 **Results and Discussion**

260 **Sample preparation and LC-HRMS analysis**

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32 During method set up two urine:water dilution factors, namely 1:5 and 1:10, were tested for dilute&shoot approach. The
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264 1:10 dilution gave better results in terms of SSE and was selected for the analysis. The IAC purification step was
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267 introduced in order to reduce the LOD/LOQ values. The employed IAC contains specific antibodies to aflatoxins B₁,
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269 B₂, G₁, G₂ and M₁, no specific information were given by the supplier for AFB₁-N⁷-Guanine. To evaluate the risk of
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269 adduct loss during purification, the synthesized adduct was applied to the IAC, eluted according to method protocol and
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270 the presence of the adduct was confirmed by LC-HRMS identification.
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273 HRMS conditions were set by direct infusion of standard solution for AFB₁ and AFM₁, while for AFB₁-N⁷-Guanine the
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275 selection of collision energy and specific fragments were guided by the work of Walton et al. (2001). AFB₁ produces
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278 sodiated adduct in a non negligible amount when compared with protonated adduct during electrospray ionization step,
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270 although AFB₁ is unlikely produce ammonium adduct, the presence of the ammonium in the mobile phase suppresses
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279 the sodiated adduct in favour of the protonated one. This is the reason for the presence in the mobile phases of formic
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272 acid and ammonium formate. For quantitative purpose the protonated adducts were selected, for AFM₁, since it was not
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273 possible to reduce the sodiated adduct production by varying source parameters, the sum of the protonated and sodiated
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275 adduct was considered.

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Validation parameters

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277 Linearity was checked in the working range by the lack-of-fit test based on the analysis of variance (F test with p value
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279 <0.05) and the plot of the residual values randomly distributed around zero, confirming the linearity. During routine
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279 analytical sessions an $R^2 > 0.990$ was set as a criterion for calibration curve acceptability. In Table 3 the calibration
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281 curve ranges, the amount of ISTD added to each calibration level and the correlation coefficients (R^2) obtained for each
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281 mycotoxin/matrix combination are reported.

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283 The performance characteristics, in terms of R_A , SSE and R_E , are summarized in Table 4 together with LOD and LOQ
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283 values and the working range of the two analytical procedures. Both methods may be applied for quantitative analysis
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285 of AFB₁ and AFM₁ as well as for the evaluation of presence/absence of the AFB₁-N⁷-Guanine adduct. The dilute&shoot
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285 method is characterized by higher LOD and LOQ values when compared with the IAC clean-up method, but on the
286
287 other hand the dilute&shoot approach is very quick and characterized by a conservative approach with respect to the
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288 sample, giving the possibility of a retrospective analysis on the acquired data. Due to the absence of a sample pre-
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290 treatment only matrix effect, in terms of SSE, and precision, in terms of RSD_{LR} , were evaluated during dilute&shoot
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290 method validation. SSE percentages are very close to 100% due to the dilution applied to the urine sample; method
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291 precision was assessed by performing 8 independent analyses at the LOQ level. The IAC clean-up method was fully
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291 validated, trueness was evaluated in terms of apparent recovery (R_A) while precision was assessed by laboratory
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292 reproducibility RSD_{LR} measures. Although the IAC clean-up, which is a very selective approach, was used, the
37
293 influence of the matrix was also evaluated, and the percentages of SSE for AFB₁ and AFM₁ found confirmed that the
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294 influence of the matrix on the instrumental response is very limited.

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295 LOD and LOQ of analytical methods always represent a challenge being the bottleneck for the reliability of the
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296 analytical results and also for the further processing of the findings (i.e. data mining). Modern HRMS instruments
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297 makes it possible to reach high sensitivities with low detection limits, and especially when methods are targeted, good
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298 benchmarks can be achieved. Among the most recent studies on the biomonitoring of aflatoxins, the lowest values for
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299 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
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300 (Giolo et al. 2012; Romero et al. 2010). Although these values represent a gold standard benchmark, they are not
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301 covered by the strict performance requirements of accuracy, which instead were met at 10 pg/mL_{urine} (Giolo et al. 2012)
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302 and 4 pg/mL_{urine} (Romero et al. 2010). Thus, the LOD/LOQ values obtained in the IAC method, validated under strict
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303 performances, are in alignment with the findings in other biomonitoring works for AFM₁. Notwithstanding, all the

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304 positive samples were in the range of values between LOD and LOQ, revealing the crucial need to stress the method to
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309 reach lower levels. In conclusion, the general validation results obtained in this study are considered satisfactory either
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306 for screening and for *confirmation* and the method is considered to suit for the production accurate data for
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307 biomonitoring purposes.

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Analytical results

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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 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). 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 dataset 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&shoot method, through which none of the sample showed a measurable level of AFB₁ or AFM₁, including AFB₁-N⁷-Guanine which 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 IAC clean-up to clean and concentrate the urine sample. By using this method for reprocessing the urine samples, AFB₁ and its adduct were not detected, AFM₁, 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

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335 should be noted that when values reported for AFM₁ are below the LOQ they were considered as affected by a standard
336 uncertainty higher than 25%, which was the performance criteria set for maximum standard uncertainty for the LOQ.
337 The LB-UB values reflect the optimistic and pessimistic scenario range of possible mean values.
338 Figure 1 shows the data trend for AFM₁ in urine for both groups, Monday and Friday sampling. On the left side LB
339 substitution method was applied, due to the high number of non-detected (87%), box plot is flattened to zero. On the
340 right side the box plot reports all the positive values are reported. The band inside the box is the second quartile (P50,
341 median). Dots indicates suspected outliers. Whiskers are set from minimum to maximum value. First and third quartiles
342 (P25 and P75) can be found at the bottom and the top of the box, respectively.
343 No statistical difference for AFM₁ was observed between Monday and Friday samples in each group (exposed and non-
344 exposed workers). To note that among the positive results two individuals of the exposed workers group showed AFM₁
345 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
346 for each individual, respectively. Further statistical analyses were performed merging data of Monday and Friday data
347 (63 analyses for exposed workers group and 57 for non-exposed workers group). Eight samples (13%) resulted positive
348 in the workers' group where the highest contaminated sample was found (10.5 pg/mL_{urine}); six samples (11%) were
349 positive in the control group, the higher detected value was 4.1 pg/mL_{urine}. In order to find differences among the
350 positive values found in workers and control group, a Wilcoxon rank-sum test was performed but no statistical
351 significances were highlighted; even exploring the two days of urine delivery, no differences were highlighted.
352 The absence of AFB₁ and its adduct, together with the absence of a statistical difference when the mean values of AFM₁
353 for workers and control groups were compared, suggests that in this specific setting, no professional exposure occurs.
354 Moreover, considering the very low level of AFM₁ in the collected urine samples, also the contribution from the diet to
355 the overall exposure is to be considered negligible.

356 **Conclusions**

357 This study presents a method, performed by a high-resolution mass spectrometry (LC-HRMS) technique, to detect
358 AFB₁ and aflatoxin biomarkers, namely AFM₁ and aflatoxin-N7-Guanine. If compared with the previous study
359 conducted in 2014 (Ferri et al. 2017), the present work tackles with an improvement of method sensitivity (1.5
360 pg/mL_{urine} versus 25 pg/mL_{urine} in Ferri et al. (2017). High percentages of workers positive for AFB₁ were reported in
361 several studies conducted in EU (Portugal, Italy, Denmark) (Olsen et al. 1988; Viegas et al. 2018; Ferri et al. 2017)
362 emphasizing that occupational exposure might be critical in certain settings, such as feed plants, in which indoor areas
363 can be conducive of highly contaminated dust particles. In this study, morning urine samples were collected during one
364 working week from a cohort of occupational exposed workers of a feed company and from a control group. The
365 presence of only one positive sample of AFB₁ and the lack of statistical difference between mean values of AFM₁ in

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366 workers and control groups, suggests that in this specific setting, the primary route of exposure to AFs is more likely to
369 be attributed to the diet and not to the respiratory route when inhalation or dermal contact of aerosolized contaminated
370 dusts occur. However, the attention and focus to AFs can't never be reduced to a no-risk situation, since, due to the
371 direct correlation between aflatoxins occurrence and climate changes, a systematic monitoring of the health status of
372 citizen (including workers) potentially exposed to dusts contaminated by these toxic compounds, has to be duly
373 undertaken.

374 **Compliance with ethical standard**

375
376 The study has been approved by the Ethical Committee of Reggio Emilia Province and performed in accordance with
377 the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

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380 **Conflict of Interest** The authors declare no conflict of interest.

381 **Acknowledgements**

382
383 This research was funded by EFSA: GP/EFSA/AFSCO/2017/05PERFORMANCE: International Conference "The
384 Burden of Mycotoxins on animal and human health" and Research Project "Biomonitoring data as a tool for assessing
385 aflatoxin B₁ exposure of workers – BIODAF". Sole responsibility lies with the author and the Authority is not
386 responsible for any use that may be made of the information contained therein.

387
388 **Gabriele Moracci, Paola De Santis, Maria Cristina Barea Toscan and Giuliana Verrone (Istituto Superiore di**
389 **Sanità), and Massimo Magnani (Servizio Prevenzione Sicurezza Ambienti di Lavoro) are acknowledged for their**
390 **technical assistance.**

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Figure 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).

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

<u>Volunteers</u>	<u>Number of subjects</u>	<u>Mean age (range); years</u>	<u>Mean body weight (range); kg</u>
<u>Workers group</u>	32	53 (32–65)	80.1 (62–99)

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<u>Controls group</u>	<u>29</u>	<u>48 (33–63)</u>	<u>83.4 (64–125)</u>
<u>Total</u>	<u>61</u>	<u>=</u>	<u>=</u>

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

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<u>Compound</u>	<u>Chemical formula</u>	<u>Precursor ion (m/z) [M+H]⁺</u>	<u>Fragment (m/z)</u>	<u>NCE^a</u>
<u>AFB₁</u>	<u>C₁₇H₁₂O₆</u>	<u>313.07066</u>	<u>285.07571; 241.04952</u>	<u>25</u>
<u>¹³C₁₇-AFB₁</u>	<u>C₁₇H₁₂O₆</u>	<u>330.12770</u>	<u>-</u>	<u>-</u>
<u>AFM₁</u>	<u>C₁₇H₁₂O₇</u>	<u>329.06558 + 351.04752^b</u>	<u>273.07538; 229.04937</u>	<u>27</u>
<u>¹³C₁₇-AFM₁</u>	<u>C₁₇H₁₂O₇</u>	<u>346.12261 + 368.10456^b</u>	<u>-</u>	<u>-</u>
<u>AFB₁-N⁷-Guanine</u>	<u>C₂₂H₁₇N₅O₈</u>	<u>480.11499</u>	<u>152.05678, 165.05499</u>	<u>40; 90</u>

^aNormalised Collision Energy; ^b[M+Na]⁺

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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	U-[¹³C₁₇]-AFB₁	U-[¹³C₁₇]-AFM₁	AFB₁	AFM₁
Dilute&shoot	5—100	10—200	10	20	0.9965 (0.04)	0.9967 (0.20)
IAC	2.5—50	5—100	50	100	0.9973 (0.09)	0.9976 (0.10)

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Table 4 Performance characteristics obtained during validation for AFB₁ and AFM₁ in urine with ~~d~~Dilute&shoot -and Shoot-and IAC clean-up methods

	<u>Dilute&shoot method</u>		<u>IAC clean-up method</u>	
	<u>AFB₁</u>	<u>AFM₁</u>	<u>AFB₁</u>	<u>AFM₁</u>
<u>LOD (pg/mL_{urine})</u>	<u>20</u>	<u>40</u>	<u>0.8</u>	<u>1.5</u>
<u>LOQ (pg/mL_{urine})</u>	<u>50</u>	<u>100</u>	<u>2.5</u>	<u>5.0</u>
<u>Working range (pg/mL_{urine})</u>	<u>50.0—1000.0</u>	<u>100.0—2000.0</u>	<u>2.5—25.0</u>	<u>5.0—50.0</u>
<u>RA (%)</u>	<u>=</u>	<u>=</u>	<u>101</u>	<u>98</u>
<u>RE (%)</u>	<u>=</u>	<u>=</u>	<u>97</u>	<u>92</u>
<u>SSE (%)</u>	<u>82</u>	<u>111</u>	<u>104</u>	<u>107</u>
<u>RSD_r (%)</u>	<u>8</u>	<u>11</u>	<u>6</u>	<u>12</u>

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Table 5. Percentage of positive samples, maximum values found and mean values (LB-UB) for AFM₁ 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

^aPositive: values above LOD
^bMax: maximum value
^cvalue below the LOQ

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

	Positive ^a (%)	Max ^b (pg/mL _{urine})	Mean (LB-UB) (pg/mL _{urine})
Monday and Friday; subjects (n=57)	11	4.1	0.3-4.8
Monday; subjects (n=29)	7	2.8 ^c	0.2-4.8
Friday; subjects (n=28)	14	4.1 ^c	0.4-4.7

^aPositive: values above LOD

^bMax: maximum value

^cvalue below the LOQ

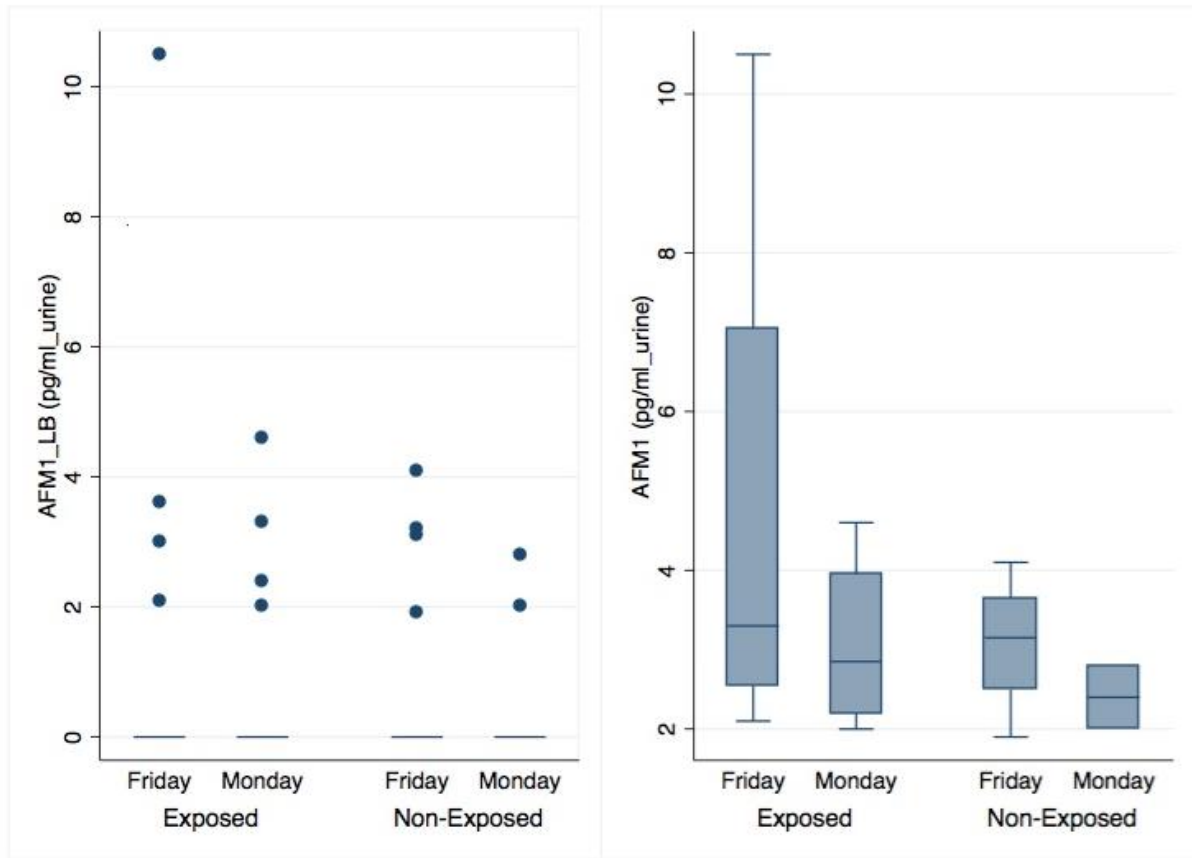
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3RD REV (letter 19/12/2019)

Reviewers' comments:

1. Delete heading "Conclusions", this journal has no such section. Concluding remarks should be put directly after end of results and discussion. [DONE](#)
2. Do not use justified text, but type left-aligned throughout the manuscript without automated hyphenation (?). Then please check if some typos (extra space etc) become visible and correct im necessary. [DONE](#)
3. Move "Acknowledgements" section directly before the reference list section [DONE](#)
4. Temperature and other units: consistently add one space between number and unit, for example "2 °C". Only % should be placed directly at the number, for example "98.3%" [DONE](#)
5. Consistently use only SI units, for example L119 100 mmol/L phosphate, and look for other occurrences as well. [DONE](#)
6. L124 what does "Guanine dissolved in 0.1 N HCl (0.32 µmoles)" mean? a "mol" has no plural, just numbers. Replace N with mol/L [DONE](#)
7. Check reference list again carefully and correct/modify according to examples as given in the instructions for authors of this journal (for example, EU regulations, EC-regulations, FAO documents). Remove issue numbers in brackets, not necessary. At present, reference list is full of typos etc. NO capitals in reference text except first word. [DONE](#)
8. Figure legends should be placed on a separate page not directly at figures. There is a supplementary file in the first revision named capture list but this appears to be empty. Please place list of figure captions after the references on a separate page in the manuscript, before the tables. [DONE](#)
9. Tables: just 3 horizontal lines, one above and one below the main table body, and one separating the first parameter row from the data entries. No extra horizontal lines in the main table body. Tables 1 and 2 are ok. [DONE](#)

Figure 1



Dear Editor

Please find enclosed the revision of the research paper entitled “Optimization and validation of a LC-HRMS method for aflatoxins determination in urine samples” done following the comments from reviewers (letter 19 Dec 2019).

I thank you again for the opportunity to publish the manuscript in your respected journal.

Sincerely yours

Barbara De Santis