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Characterization and validation of sampling and analytical methods for mycotoxins in workplace air

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Mycotoxins are produced by certain plant or foodstuff moulds under growing, transport or storage conditions. They are toxic for humans and animals, some are carcinogenic. Methods to monitor occupational exposure to seven of the most frequently occurring airborne mycotoxins have been characterized and validated. Experimental aerosols have been generated from naturally contaminated particles for sampler evaluation. Air samples were collected on foam pads, using the CIP 10 personal aerosol sampler with its inhalable health-related aerosol fraction selector. The samples were subsequently solvent extracted from the sampling media, cleaned using immunoaffinity (IA) columns and analyzed by liquid chromatography with fluorescence detection. Ochratoxin A (OTA) or fumonisin and aflatoxin derivatives were detected and quantified. The quantification limits were 0.015 ng m^{-3} OTA, 1 ng m^{-3} fumonisins or 0.5 pg m^{-3} aflatoxins, with a minimum dust concentration level of 1 mg m^{-3} and a 4800 L air volume sampling. The methods were successfully applied to field measurements, which confirmed that workers could be exposed when handling contaminated materials. It was observed that airborne particles may be more contaminated than the bulk material itself. The validated methods have measuring ranges fully adapted to the concentrations found in the workplace. Their performance meets the general requirements laid down for chemical agent measurement procedures, with an expanded uncertainty less than 50% for most mycotoxins. The analytical uncertainty, comprised between 14 and 24%, was quite satisfactory given the low mycotoxin amounts, when compared to the food benchmarks. The methods are now user-friendly enough to be adopted for personal workplace sampling. They will later allow for mycotoxin occupational risk assessment, as only very few quantitative data have been available till now.

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Environmental impact

This paper presents an approach to routine monitoring of airborne mycotoxins. The presence of these toxic substances in foodstuff is strictly controlled for consumer safety, but occupational exposure remains largely unknown. An aerosol of contaminated particles can be formed during grain, flour and bean handling. The methods that we have characterized and validated to seven of the most frequently occurring mycotoxins are now available for field measurements. It will thus be possible to investigate a wide range of dusty environments and generate new data. This work is an important first step aimed at improving knowledge of the risks due to mycotoxin inhalation.

Introduction

Mycotoxins are chemical substances produced by moulds that can colonize cereals, nuts, beans, seeds and grapes due to particular environmental, transport or storage conditions. Any crop is a possible target for mould growth and mycotoxin formation, whether in the field or after harvesting.[1–7] Mycotoxins can be found in cereals and any foodstuff due to the use of contaminated ingredients. More than 300 mycotoxins have been identified and some poorly described, but only 20 of them are normally found in food and feed at levels that are considered to be of concern.[8] Among these, aflatoxins are produced by at least three species of *Aspergillus*, ochratoxin A (OTA) is produced by species that belong mainly to the *Aspergillus* and *Penicillium* genera and fumonisins are *Fusarium* toxins. Mycotoxins have also been studied in water-damaged buildings, as secondary metabolites of *Aspergillus, Streptomyces, Penicillium, Cladosporium* and *Stachybotrys* predominantly growing on cellulose-derived material.[9–14] The absence of any visible mould does not guarantee freedom from toxins as the mould may have already died out leaving the toxin intact. Most mycotoxins being chemically stable and highly resistant to temperature treatments, full destruction during conventional food or feed production does not occur. Not only do these substances have a considerable agro-economic impact, they are

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also a threat to human and animal health through the ingestion of contaminated food or feed. Toxic for the kidneys, liver and immune system, they are also carcinogenic, possibly carcinogenic, genotoxic, or can cause birth defects and even death. While its acute toxicity is low, fumonisin B_1, for example, has been found to be hepatotoxic and nephrotoxic for every animal species tested and possibly carcinogenic to humans (IARC Group 2B).[5] Legislation has been established worldwide, with maximum limits for different foodstuffs. In the European Union, Commission Regulation (EC) no. 472/2002 sets for example a maximum OTA level for unprocessed cereal equal to 5 μg kg^{-1}. Similarly, Commission Regulation (EC) no. 1126/2007 sets a maximum Fumonisin B1 and B2 level for maize-based breakfast cereals equal to 800 μg kg^{-1}.[15] Besides the known and well-investigated alimentary sources, the problem is also arising from occupational exposure. Workers are probably exposed whenever they store, load, handle or mill contaminated materials. These operations include farming activities, grain working, commercial storage, obviously the food and feed industry, but also food contaminant analysis and waste treatment. Yet, until a few years ago, hardly any studies had examined the impact of mycotoxins in workplace air.[9,16–22] The measuring techniques were sometimes not sensitive enough, whether measuring mould or mycotoxin levels in foodstuffs, in settled dust or in air. The mycotoxin concentration results were so heterogeneous that it was still impossible in 2008 to reach a conclusion concerning their significance in worker exposure and diseases. The health effects associated with inhaled mycotoxins had been revealed through several reports: immunotoxic effects on the rat due to inhalation of OTA contaminated air, a case of acute renal failure, and ochratoxin found in house dust correlated with ochratoxin poisoning in animals.[23–25] Some data even suggested that another *Fusarium* toxin, desoxynivalenol (DON), was more toxic to the adult female mouse when nasally exposed than when orally exposed, with effects on tissue distribution and proinflammatory cytokine induction.[26] The authors publishing at that time concluded that exposure to airborne mycotoxins might represent a risk for the development of allergies.[27] However, additional data were still necessary to evaluate the health risk due to mycotoxin inhalation during occupational activities.

This paper discusses the sampling and analytical methods that we characterized for seven of the most frequently occurring mycotoxins in airborne particles. The methods had to integrate aerosol sampling and subsequent mycotoxin analysis of the particles collected. The sampler evaluation was performed on experimental atmospheres and the methods were studied regarding reproducibility, user-friendliness and reliability, all being necessary for personal workplace sampling.

Selection of the personal workplace sampler

Before describing the study and the methods, we highlight here the different aspects we took into account when selecting a personal aerosol sampler. Many available bioaerosol samplers had already been tested and compared by Griffiths *et al.* and Fabries *et al.* regarding their collection efficiency, bioefficiency and assay efficiency for aeroallergens and aeropathogens.[28–30] It was shown that samplers should collect the inhalable dust fraction, which approximates to the airborne particles that enter the nose and mouth during breathing and can therefore settle in the respiratory tract. We also took into consideration several other parameters associated with the monitoring of these types of airborne substances. It had already been suggested that the direct measurement of airborne mycotoxins was a more relevant assessment approach. Free bacteria, microbes or moulds, at least those taking the form of viable fungal cells, spores or fragments, did not therefore have to be measured. No biological analysis, culture, count or identification was needed. In addition, the liquid collectors or impingers were not only less convenient to handle, transport and process during occupational sampling but they further diluted the air samples in a collection fluid, possibly making them unsuitable for the following analytical step. High sensitivity (at the ng m^{-3} mycotoxin level) was indeed necessary as, given the regulatory control limits required in the food industry, the airborne concentrations were generally expected to be very low. The limited existing data seemed to indicate that the concentration levels in air could even be lower, even for sites chosen for their susceptibility to high mycotoxin contamination (0.189 and 0.080 ng m^{-3} on average for aflatoxins G2 and B1,[16] less than 8.304, 0.04, 0.029, 0.04 and 0.131 ng m^{-3} for OTA, aflatoxins B1, B2, G1 and G2 respectively,[21] less than 0.04 and 0.013 ng m^{-3} for OTA and aflatoxins[22]). Any sampler operating with too low a flow rate, such as the 1 to 2 mL min^{-1} filter-in-cassette, was consequently discarded from our selection. The study was targeted at personal occupational measurements, therefore on-line or real-time sampling was unsuitable. The reliability of 8 hour sampling was a key parameter for the sampler. Our choice was finally dictated by convenience: a small and lightweight personal sampler, commonly used in industry for measuring dust, fibers or moulds in "real-life" sampling conditions, should be part of a user-friendly method.

Experimental

Chemicals

The mycotoxin primary standards were 10 μg mL^{-1} of OTA in an acetonitrile solution (Sigma 34037, Sigma-Aldrich Chemie, Germany), 50 μg mL^{-1} of both fumonisin B1 and B2 in an acetonitrile–water mixture (Romer S02003, Austria), and 250 ng mL^{-1} of each aflatoxin B1, B2, G1, G2 in acetonitrile (Libios STD AFBG 250A, Bully, France), all used as recommended by the suppliers. Acetonitrile (RS for isocratic HPLC, Carlo-Erba Reagents, France), methanol (Lichrosolv for HPLC, Merck, Germany), acetic acid (100% Norm, VWR-International, France), nitric acid (Rectapur, Prolabo, France), phosphoric acid (H_3PO_4 85%), sodium dihydrogen phosphate NaH_2PO_4, and sodium borate decahydrate $Na_2B_4O_7$ (ReagentPlus®, ≥99.5%) from Sigma-Aldrich Chemie (Germany), *o*-phthaldialdehyde (≥97% for HPLC), 2-mercaptoethanol (≥99.0%), potassium bromide (Purum p.a., ≥99.5%) and boron trifluoride BF_3 (10–20% in methanol) from Sigma-Aldrich Chimie (France)

were used. Purified water (18 MΩ cm) was obtained from an Academic MilliQ model water purification unit (Millipore, EMD Millipore Corporation, Billerica, MA, USA). The extraction solvents were mixtures of acetonitrile and water (60/40 v/v), methanol and water (75/25 v/v) or methanol and water (60/40 v/v). The elution solvents were methanol–acetic acid (98/2), methanol–water (50/50) or pure methanol. The phosphate buffer saline solution (PBS) was prepared from tablets purchased from Libios (Bully, France) and purified water. The OPA–MCE reagent was obtained from the *o*-phthaldialdehyde reagent (40 mg of *o*-phthaldialdehyde in 1 mL methanol), diluted with 5 mL of 0.1 M $Na_2B_4O_7$, added to 50 μL of 2-mercaptoethanol and used within 24 hours.

Instrumentation

Multipurpose generator system. The atmosphere generation system (photo in Table 1) was described by Freville *et al.*[31] The PALAS RBG 1000 rotating brush aerosol generator equipped with a stainless steel brush was located at the end of a dust-filled cylinder. The concentration was held constant by setting the piston rise speed in the cylinder. This conventional type of generator continuously dispersed up to 10 g of dust and was coupled to the exposure chamber. The aerosol injection system was designed to create strong turbulence in order to homogenize the aerosol; a straight section then stabilized the airflow. A Dustscan™ nephelometer monitored the changes in aerosol particulate concentration. A computer application was installed to start or stop the experiments and record the generation process in real time. Air flow rates, pressure, humidity and aerosol particulate concentration data were continuously stored and displayed. The particle size distribution in the aerosol could be also determined with a Marple™ multi-stage personal cascade impactor.

Matrix sifting. A sieve of woven wire cloth (200 mm diameter ring, 100 μm square mesh) was used to manually sift powdery flour, as described in Standard NF ISO 2591-1.[32] The particle size distribution of the solid matrices was then ascertained with a Malvern™ Mastersizer X.

CIP-10 personal aerosol sampler. The CIP-10 personal aerosol sampler (ARELCO, France) is based on a rotating filter cup designed by Courbon *et al.*[33] The particles are driven by centrifugal force towards the rotating collection cup equipped with a porous polyurethane foam filter. The new version of the particle-size selector for the inhalable aerosol fraction (CIP 10-I sampling unit), designed and patented by INRS, was used.[34,35] The flow rate of the CIP 10-I sampler was set to 10 L min^{-1} and calibrated on a test rig using pressure drop compensation. Its stability was estimated by checking the cup rotation speed with an ARC 8527 tachometer (ARELCO, France).

Weighing. An AX26 balance (Mettler Toledo, France), accurate to the microgram level, was used in a weighing room with controlled temperature and hygrometry conditions.

Immunoaffinity (IA) columns. Ochraprep® P14, FumoniPrep® P31 and AflaPrep® P07 (R-Biopharm, France), Purifast AEMG 1060-3 and AFBG-1 (Libios, France), contain immobilized antibodies specific to one mycotoxin or a family of mycotoxins. They were used as recommended by the supplier (storage temperature before use, type and volume of conditioning, washing and extraction solvents, elution speed, backflush technique).

Chromatographic system. The liquid chromatography (LC) system consisted of a Series 200 binary pump, a Series 200 vacuum degasser, a Series 200 auto-sampler operating at 10 °C, a Series 200 column oven heated to 40 °C, and a Series 200 fluorescence detector (FL detector), all manufactured by Perkin Elmer. The separation column was either an Alltech Alltima™ HP Reversed-Phase C18 (5 μm, 150 mm, 3 mm I.D.) or a Grace Davidson Discovery Sciences GraceSmart™ RP18 (5 μm, 250 mm, 4.6 mm I.D.). The samples were introduced using a syringe injection valve with a 10 to 200 μL adjustable loop. The data were acquired and analyzed with Perkin Elmer Chromera software. LC conditions were optimized for each type of mycotoxin.

For the OTA analysis: a mixture of acetonitrile–water–acetic acid (49.5/49.5/1 v/v/v) as the mobile phase, a flow rate of 0.5 mL min^{-1}, the Alltima™ HP column, a 80 μL injection volume, and the FL detector set to an excitation wavelength (λ_{exc}) of 330 nm and an emission wavelength (λ_{em}) of 470 nm.

Table 1 Conditions of aerosol generation from barley flour

		Particle sizes
In the exposure chamber (here open), 20 samplers could operate simultaneously on an alternating rotating system (1 turn per min)		
	Contaminated barley flour	Ranging from 2 to 500 μm, mean 337.2 μm (s = 3.9)
	Sifted flour	<100 μm. Coarse particles, which could settle in the system, had been eliminated through a sieve. They should neither be sampled nor inhaled in a real aerosol
	Aerosol	90% of the particles with an aerodynamic diameter (D_{ae}) <20 μm, mean 9.96 μm (s = 2.33)

For the fumonisin analysis: a mixture of methanol–sodium dihydrogen phosphate 0.1 M (75/25 v/v) as the mobile phase adjusted to pH 3.35 with phosphoric acid (85%), a flow rate of 0.5 mL min^{-1}, the Alltima™ HP column, and the FL detector set to 335 nm and 440 nm. The following pre-column reaction program was applied to the auto-sampler: 10 μL OPA–MCE reagent added to 120 μL of sample solution and mixed; 80 μL injected into the LC system within 3 min.

For the aflatoxin analysis: a mixture of water, methanol and acetonitrile (3/1/1 v/v/v) with 119 mg of potassium bromide and 350 μL of 4 M nitric acid for 1 L of the mobile phase, a flow rate of 1 mL min^{-1}, the GraceSmart™ column, a 80 μL injection volume, a post-column reaction device (Kobra Cell®, R-Biopharm France), and the FL detector set to 365 nm and 435 nm.

Aerosol generation and sampling

Generation matrices. Dusty atmospheres were generated from naturally contaminated barley or maize flour. The contaminated matrices were purchased from food analysis laboratories and mycotoxin interlaboratory ring test organizers. To be used as the generation matrix, powdery flour was manually sifted to eliminate coarse particles which could have settled in the generator system. The particle sizes are given in Table 1 for the contaminated barley flour and the sifted flour used in the generator system. The mycotoxin concentration for the sifted matrix was measured by high-performance liquid chromatography (HPLC), according to NF EN 14132:2009.[36]

Mycotoxin aerosol generation and sampling of airborne particles. The particle size distribution in the aerosol (reported in Table 1) was determined with the multi-stage cascade impactor. The mycotoxin contamination in the different size fractions was not measured, given that less than 0.5 mg of dust was collected on each grease-coated filter.

Sampling was performed in the exposure chamber using 15 CIP-10 samplers, symmetrically positioned on the rotating sampling rig and operating simultaneously. Their flow stability was estimated by checking the cup rotation speed with the tachometer before and after each sampling series. The difference from the calibrated speed had to be less than 5%. Eleven series of samples were generated: seven series generated from contaminated barley and four series generated from contaminated maize. In the case of barley flour matrices, the amount of dust for an 8 h sampling time corresponded to 0.4- to 1-fold the occupational limit value for an inhalable dust. The maize flour appeared unsuited to the generation of dust concentrations higher than 0.3-fold the occupational limit value, as its physical characteristics involved the deposition of agglomerated particles. The generation duration ranged from 100 to 213 min, limited by the length of the piston path in the aerosol generator cylinder together with its rise speed. The sampled air volumes therefore ranged from 1000 L to 2130 L. The average value of the mass concentration of the suspended dust in the aerosol was ascertained by weighing the dust collected. Five unused collection substrates for 15 collected samples were taken as blanks. They were systematically handled and analyzed like the other samples.

Validation experiments. Some experimental trials were performed to check whether our generation and sampling conditions were concordant with those already validated for the multipurpose generator system and the CIP 10-I sampler. In our study, the mass concentration of the suspended dust was monitored in real time, but the average value was always ascertained by weighing the dust collected. P. Görner *et al.* demonstrated the reliability of CIP 10-I samplers to measure inhalable aerosol,[37–39] their performance being compared to the ACGIH,[40] CEN[41] and ISO[42] specifications. Our tests focused on the dispersion of the quantity of dust deposited on the foam pads to assess the convenience of CIP 10-I sampling for mycotoxin contaminated cereal particles compared to the particles already used to validate the sampler.

Spiking technique

A homogeneous suspension of contaminated flour blended for 10 hours was prepared in purified water. One or two mL aliquots were taken from the middle of the solution and injected onto the foam pads previously weighed in their cup. The spiked samples were then dried in an oven heated to 50 °C and weighed on a balance accurate to the microgram level. The amount of dust spiked on each substrate could be calculated from the weight difference between the spiked sample and the same foam pad in its cup before spiking. Weight differences were compared to those of the spiking blanks (samples spiked only with water) and corrected for weight variations in the weighing blanks (non-spiked samples).

Particle recovery and mycotoxin analysis

Measurement of particle mass concentration. To ascertain the quantity of contaminated dust collected, each foam pad was weighed in its cup before and after sampling. To ensure precision down to the lowest weights possible, we followed a validated in-house protocol: prior to weighing, all the samples and blanks were dried in an oven at 50 °C for at least 4 h and left overnight in the weighing room. Weight difference was corrected for weight variation in the blanks.

Mycotoxin analysis. A number of analytical techniques for mycotoxins had already been comprehensively discussed by N.W. Turner *et al.*[43] In our study, the analyses were performed with HPLC, the most widely used technique, as noted by N.W. Turner, and were in accordance with the CEN standards and criteria.[36,44–46] Specific analytical difficulties were also considered, including greater interference of certain substances compared to a lower analyte concentration level in air samples than in food samples. The protocol to analyze a sampling substrate is presented below and illustrated in Fig. 1.

The mycotoxins were ultrasonically extracted from the sampling foam pad with 10 mL of a solvent mixture; the cup deposits were recovered with 3 mL of the extraction solvent. The sample extract was transferred to a flask and diluted with PBS solution to ensure a maximum organic solvent content. After magnetic stirring, the solutions were filtered through a cone-shaped paper filter. The filtered extract was applied to an IA column (previously warmed up to room temperature,

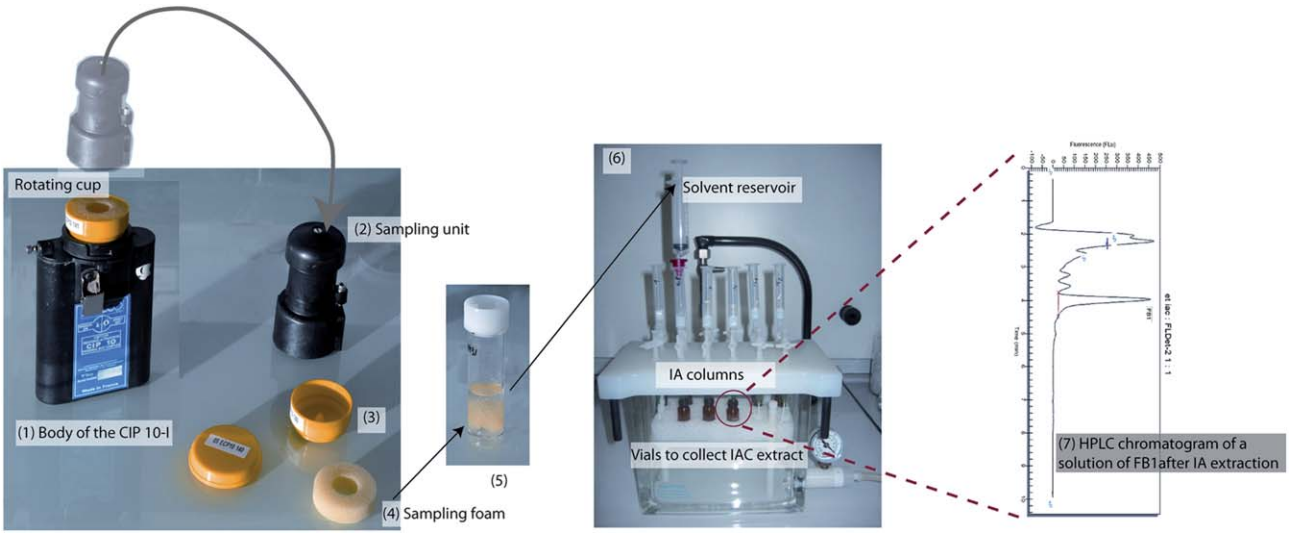

Fig. 1 View of the CIP 10-I (1) during the laboratory analysis, its sampling unit dismantled (2), the rotating cup open (3) and the sampling foam removed from the cup (4). After the solvent extraction (5), the sample is applied to the IAC system (6). The IAC extract is then analyzed by HPLC-FLD (7).

conditioned, washed and equipped with an appropriate solvent reservoir) specifically designed for one type of mycotoxin. A rinsing step with water or PBS removed any interference. The mycotoxins were eluted from the column with methanol (acidified for OTA, pure or mixed with water if not later concentrated for the other mycotoxins). The IA eluate was diluted or concentrated (evaporated to dryness under a gentle stream of nitrogen and redissolved in a reduced volume of mobile phase). An aliquot was then introduced into an injection vial. The subsequent analysis was performed by HPLC with direct fluorescence detection (for OTA) or after pre-column (for fumonisins) or post-column (for aflatoxins) derivatization. The OPA–MCE reagent–fumonisin reaction yielded products with a maximum fluorescence on analyzing the reaction solution immediately. The fluorescence began to decrease after a few minutes and the reproducible time between adding the OPA reagent and injecting it into the LC system was critical. The calibrant solutions were prepared from the primary standards and diluted (in an acetonitrile–water–PBS buffer mixture for OTA, in a methanol–water–PBS buffer mixture for the fumonisins, and in a methanol–water mixture for the aflatoxins respectively). They ranged from approximately 5 to 125 pg mL^{-1} for OTA, 4 to 100 ng mL^{-1} for fumonisin and 0.6 to 60 pg mL^{-1} for each aflatoxin. These calibrant solutions were subsequently analyzed with exactly the same protocol (IA column purification, later dilution or concentration, HPLC) as the samples, one calibrant solution series for each set of samples to be analyzed. A calibration curve using the linear least-squares fit technique was calculated. Quantitative determination was performed by comparing the peak area for the sample to the corresponding calibration curve. The laboratory blanks were prepared from foam pads in their cup using exactly the same protocol (extraction, dilution, IA purification, IA subsequent concentration). The mycotoxin concentration in the injection volume, then the amount of mycotoxin extracted on the IA column, and finally the airborne concentration (taking into account the air volume collected) were calculated. The results were adjusted by subtraction for any positive laboratory blank. Corrections were also made, where appropriate, for the dilution introduced by the derivatization, the dilution of the IA eluate, or the nitrogen concentration. As is standard practice in occupational measurement, the field blank results were used to validate the sampling and were not taken into account in the calculations of the mycotoxin concentration.

Validation experiments. The preliminary tests focussed on recovery experiments once the dust had been extracted from the foam pads and recovery tests once the dust had been filtered through a paper cone. The solvent extraction technique, the derivatization parameters (volume of OPA–MCE reagent and reaction time), the mixture required to dissolve the concentrated dry residue (type and volume), and the LC conditions (including the test of 50, 80 and 100 μL injection volumes) were studied and optimized. The performance characteristics were then evaluated for the following parameters: mycotoxin recovery (calculation of the mean analytical recovery rate, study of the IA extraction yield and the nitrogen-concentration step yield), HPLC analysis (ten time replicated injection repeatability, instrumental limit of detection estimated as the concentration giving a signal-to-noise ratio equal to 3, analytical limit of quantification and precision as specified in Standard EN 1076: 2009) and mycotoxin stability on the sampling media.

Uncertainty estimation

The aim of the study was to characterize and optimize methods intended to monitor occupational exposure to airborne mycotoxins. There was no point in our developing too sophisticated or in-house methods that would be difficult to reproduce. In order to offer laboratories subsequently using them, the possibility of working with the same level of quality, the methods were described as precisely as possible. Each step was studied and the expanded uncertainty (U) was calculated.

Field testing

Once experimentally validated, the methods were used to assess airborne mycotoxins in workplaces. The OTA and fumonisin measurements were performed in a commercial seaport during ship unloading (I). Personal samples were collected from workers in backhoe-loaders during the transportation or transfer of maize and wheat grain. Stationary air sampling was also performed in the operator cabin where no personal protective equipment was worn while working. The inhalable airborne particle levels and OTA concentration were also measured in a factory producing liquorice powder for confectionery and tobacco (II). As aflatoxins and OTA are frequently reported as contaminants of nutmeg, black and white pepper, turmeric and cloves, and can occur in root vegetables and fruits, these mycotoxins were measured in the food industry: in a factory (III) where root chicory is baked and ground, in another (IV) where people are employed storing, weighing, milling and packaging spices for pork based products and ready-made meals, and finally in (V) where spices are used to prepare meat sausages. The sampled air volumes depended on the operations carried out in the five workplaces. To identify any contamination arising from handling in the field or during transportation and weighing, five field blanks for every 15 samples were transported to each sampling site. These were processed along with the stationary and personal samples.

Results and discussion

Aerosol generation and sampling

More than 160 air samples were generated, with dust concentrations ranging from 0.3 to 9 mg m^{-3}, ochratoxin concentrations between 0.1 and 0.5 ng m^{-3} (5- to 10-fold the maximal regulated OTA level for cereals, for example), and fumonisin B1 concentrations lower than 10 ng m^{-3} (the maximal fumonisin level regulated for milling fractions of maize with particle size $\leq$500 microns, for example). The linear function calculated between the piston rise speed and the average dust concentration value for each generation series demonstrated that the multipurpose generator system worked well and was in compliance with the conditions already validated.[31] No atmosphere was generated in the multi-purpose system with the highly toxic aflatoxin B1. Nevertheless, the sampling procedure had already been validated with OTA contaminated aerosols. As the sampling performance of the sampler does not depend on the mycotoxin itself, additional fumonisin and aflatoxin samples were produced from liquid spiking.

The heterogeneity of the collected masses was low with a relative standard deviation of 4.6%, the same level previously established with silica dust.[31] The particle collection efficiency of the rotating cup, equipped with the new particle-size selector for the inhalable aerosol fraction, was known to be >45% for particles with aerodynamic diameters from 2 μm to 60 μm in air.[39] With an allowable dust accumulation of 50 to 65 mg, this sampler was appropriate for a low mycotoxin concentration, as well as an 8 hour sampling time, even in quite dusty atmospheres. On the other hand, its 10 L min^{-1} flow-rate was also suitable for situations with a low mycotoxin exposure.

Particle recovery and mycotoxin analysis

The tests were conducted using both the experimentally generated air samples and the known spiked samples. The theoretical mycotoxin quantity could be estimated for each air sample from the following parameters: mycotoxin contamination in different size fractions of the raw material; particle sizes in the experimental aerosol and CIP10-I collection efficiency for these particle sizes.

The preliminary tests showed total mycotoxin transfer (relative standard deviation >95%) from the sampling media to the analytical solution. The filtration of highly loaded dust through a paper filter cone generated no mycotoxin loss. A total of 30 OTA samples for 2 air series, 48 fumonisin spiked samples (6 levels) and 72 aflatoxin spiked samples (4 levels) were used for the first test, while 20 OTA air samples (2 levels), 6 OTA contaminated flour samples (4 levels) and 6 fumonisin contaminated flour samples (4 levels) were used for the latter. The optimized parameters were adopted in the final method. Three extraction techniques had previously been investigated using 20 air samples loaded with 150 to 400 pg OTA, 6 OTA contaminated flour samples and 6 fumonisin contaminated flour samples. Whereas sampling foam blending was not convenient, solvent percolation through a syringe reservoir followed by a wringing motion gave results similar to ultrasonic extraction. The latter was adopted due to the need, in each case, to recover the cup deposits. Several methanol–water or acetonitrile–water mixtures were tested. The mixture giving the better yield and allowing the best chromatographic separation was chosen for the final method, for each mycotoxin. The native OTA fluorescence, when excited at a wavelength of 330 nm, allowed for direct detection after the LC column, no nitrogen concentration requirement for the IA extracts and a wide range of analytical applications. For the other substances, the need for derivatization added further analytical difficulties that had to be taken into consideration for any future occupational measurements.

The analytical characteristics are given in Table 2. Examples of the resulting LC-FL chromatograms (both for the experimental and field samples, and consistently for the blank and collected samples), are shown in Fig. 2 for OTA (a and a′), the fumonisins (b and b′) and the aflatoxins (c) with an example of the mean regression line, for each mycotoxin. Linear standard curves with a coefficient of determination $r^2 > 0.99$ were always obtained. The recovery efficiency integrating IA separation and nitrogen concentration (termed "global yield") was estimated, to assess the convenience of our analytical protocol only (at 6 levels for OTA, 12 levels for the fumonisins and 7 levels for the aflatoxins). A total of 238 OTA, 240 fumonisin and 54 aflatoxin calibrant and sample solutions were analyzed, the resulting concentration being related to the expected concentration. The nitrogen concentration yield was estimated by comparing the concentrated aliquot against the directly injected IA extract for each tested sample. The IA yield was finally calculated from

Table 2 Summary of the analytical characteristics

	OTA	Fumonisins	Aflatoxins
Repeatability of injections (RSD)	<7.5%	<6.5%	≤5%
Instrumental detection limit	25 pg mL^{-1}	10 ng mL^{-1}	From 0.7 pg mL^{-1} (B2) to 1.7 pg mL^{-1} (G1)
Analytical quantification limit	75 pg mL^{-1}	30 ng mL^{-1}	15 pg mL^{-1}
Calibration function (optimal RSD values)	5%	4%	From 2.6% (B1) to 4.8% (G1)
Mean IA yield	82%	82%	From 93% (G1) to 100% (B1, G2, B2)
Mean nitrogen concentration yield	<50% useless step	78.6%	From 91% (B1) to 100% (G1, G2, B2)
Mean analytical recovery rate	90% (CV = 10%)	100% (CV = 10%)	From 85% (B1) to 100% (B2)
Stability of the substances on the sampling media	>95%	>95%	>95%

subtracting the nitrogen concentration yield to the "global yield". The method recovery and method precision tests were carried out using the 7-level OTA generated samples, the 6-level fumonisin spiked samples and the 6-level aflatoxin samples. The analytical recovery rate was calculated as the value observed divided by the value expected. The precision of the method was calculated as a standard deviation of these results. A total of 30 OTA sampled foams at 2 levels (236 and 600 pg on the foam), 48 fumonisin spiked foams at 6 levels (5 to 240 ng) and 24 aflatoxin spiked foams at 4 levels (28 to 125 pg AfB1) were stored for one month at ambient temperature before analysis. The stability of the substances on the sampling media was shown to be quite satisfactory with total recovery after one month. Analyzing the calibration solutions with every set of samples, simultaneously and identically, allowed for the correction of any possible drift due to either a change of IA column batch or fluorescence lamp ageing. The mycotoxin determination correction was directly integrated into the regression line and, unlike the usual measurements in food matrices, the yield did not have to be taken into account. The airborne mycotoxin mass was ultimately related to the particle mass in the same cup. This estimation was designated as aerosol contamination and, when compared to the matrix contamination, was always higher. The certified 3 μg kg^{-1} OTA concentration had been checked in our laboratory for the unprocessed barley flour. The sifted flour turned out to be 15 μg kg^{-1} when analyzed. The mean experimentally estimated contamination of the dust particles in the aerosol was 50 μg kg^{-1}. Mycotoxin contamination had previously been estimated in different size fractions of the raw flour and was shown to be inversely proportional to the size fraction (15 μg kg^{-1} for particles <100 μm, 11 μg kg^{-1} for the 100–500 μm fraction and 1.4 μg kg^{-1} for particles >500 μm). In both cases, when the proportion of small particles was higher, so was the contamination, certainly due to a higher surface to volume ratio in the particles. The measuring procedure was designed for a minimum air concentration of 0.015 ng m^{-3} OTA, 1 ng m^{-3} fumonisins or 0.5 pg m^{-3} aflatoxins, with a minimum dust concentration level of 1 mg m^{-3} and a 4800 L air volume sampling.

Uncertainty estimation

The expanded uncertainty (U) was calculated by combining the sampling and analytical uncertainty components, as recommended both for food control and workplace air aerosol sampling.[47–50] To establish the confidence of the measurement results, neither a certified reference material (CMR), nor an accreditation scheme was of any help. The food CMR and the food proficiency testing were not adapted, with other purposes and method performance needs, and a different sampling method and analytical protocol. Furthermore, the air measurements require much lower analytical limits of quantification. The individual uncertainties were thus calculated from the experimental data. The uncertainty component due to analytical recovery was estimated as the maximum analytical bias. The analytical precision was calculated with data from several month lasting tests, and long-term random variations were thus taken into account. The uncertainty component associated with the calibration function was consequently included in the estimate of analytical precision and no separate uncertainty estimate was required. Optimal reproducibility values for the calibration function are given in Table 2 only for information. The elements of the calculation used to estimate the expanded uncertainty at the end of the 3 year study are presented in Table 3, when gravimetric determination of all the volumes (dilution, extraction, recovery after extraction, *etc.*) formed part of the analytical protocol. The expanded uncertainty could be estimated to be less than 50% for OTA and fumonisins. This meets the general requirements for the performance of procedures for measuring chemical agents given in Standard EN 482: 2011. Regarding analytical uncertainty only, comparison to the food benchmarks shows quite a satisfactory agreement. Reproducibility coefficients of variation from 13 to 26% are given for the determination of ochratoxin A in barley (NF EN 14132: 2009), and from 9.5 to 51% for the determination of aflatoxins in cereals (EN ISO 16050: 2011), while ours were 10% (OTA) and 19% (aflatoxins) on average.

Field testing

The results of the field trial measurements are given in Table 4. Several problems may be associated with the monitoring of mycotoxins besides the very heterogeneous distribution of toxic metabolites in stored commodities and, consequently, in products. They had to be kept in mind while sampling.[1,51] The modifications to the final protocol, described below, improved the user-friendliness of the methods. Even if the mycotoxins were known to occur in the investigated cereal or foodstuff, or could be deduced from the mandatory analyses, an identification procedure was sometimes applied, as described in the

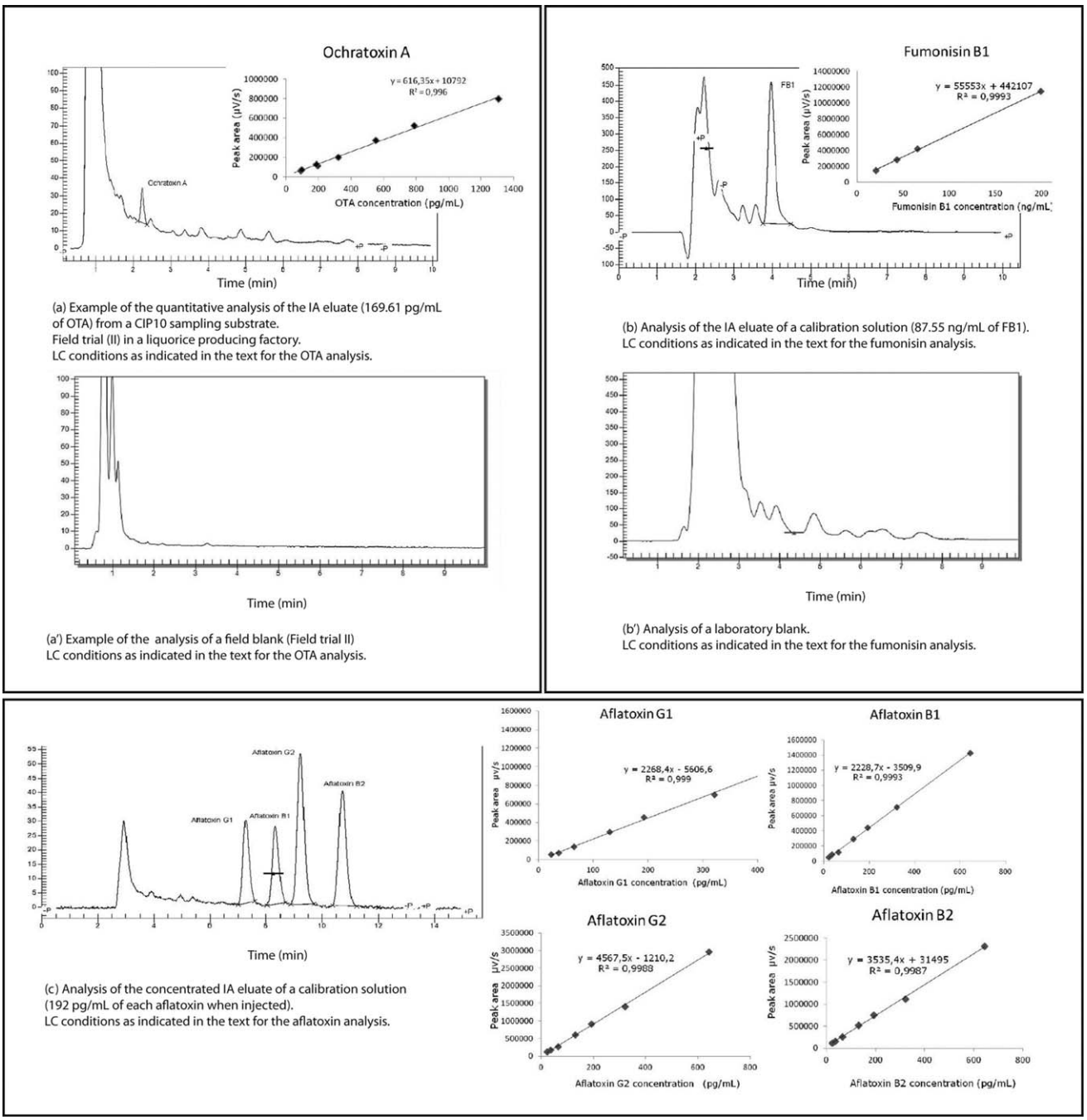

Fig. 2 The resulting LC-FL chromatograms for (a and a') OTA, (b and b') fumonisin B1 and (c) the aflatoxins (aflatoxin B1, aflatoxin B2, aflatoxin G1, aflatoxin G2), with the associated linear standard curve and mean regression line for each mycotoxin.

literature.[52] When there were many interfering peaks in the chromatograms, that of OTA could be slightly altered and prone to misinterpretation. In this case, the results were recorded only after a positive BF3-derivatization test had been applied to the corresponding samples. This type of procedure should be helpful to occupational analysts for their qualitative and quantitative determination of mycotoxins, if needs be. As only one analysis was possible for each occupational air sample, parallel stationary sampling was also systematically adopted. It should be proposed for any future occupational measurements, giving the analyst the possibility of ascertaining the mycotoxin types and approximating the concentrations. An appropriate IA extraction technique and additional treatment were then applied to the personal samples to perform the precise measurements. The quantities of bulk materials available were insufficient to analyze them according to a reliable protocol,[44] and data are not published. Regardless, the bulk material contamination level had been estimated merely for comparison with aerosol contamination and they showed that mycotoxins can accumulate in airborne dust, up to 15-fold compared to the

Table 3 Elements of the calculation used to estimate the expanded uncertainty

		Uncertainty %		
		Ochratoxin A	Fumonisins	Aflatoxins
Sampler calibration		0.35	0.35	0.35
Flow stability		5	5	5
Sampling time	(Taken to the nearest minute)		Can be disregarded	
Particle collection efficiency	(When a foam pad is used as the collection substrate)		Negligible	
Deviation from sampling convention		10	10	10
Storage		5	5	5
Transportation	(Assuming no loss during transportation)		Negligible	
Combined uncertainty for the sampling	$u_s = \sqrt{\sum_{i=1}^{7} u_{s,i}^2}$	12.25	12.25	12.25
Certified stock standard solutions		0.3	0.7	0.7
Analytical precision		10	9.5	19
Analytical recovery		10	13	15
Combined uncertainty for the analysis	$u_a = \sqrt{\sum_{i=1}^{3} u_{a,i}^2}$	14	16	24
Standard combined Uncertainty	$u_c = \sqrt{u_s^2 + u_a^2}$	18.7	20.2	27.1
Expanded uncertainty of the procedure	$U = 2 \times u_c$	37.4	40.5	54.3

Table 4 Field trial measurements

				Air samples		Airborne dust (mg m^{-3})			OTA (ng m^{-3}), fumonisins or total aflatoxins (pg m^{-3}) in air		
Sampling site	Foodstuff	Type of mycotoxin	Type of sampling	Number	Duration (min)	Mean	Median	Min–max	Mean	Median	Min–max
I	Bulk wheat	OTA	Stationary	1	106	36	—	—	0.4	—	—
I	Bulk maize	Fumonisins	Personal	1	127	27.4	—	—	55	—	—
II	Liquorice powder	OTA	Stationary	3	99–368	0.9	0.96	0.25–1.6	<0.03	—	—
		OTA	Personal	4	90–151	16.6	17.2	8.3–23.8	0.52	0.17	0.08–1.7
III	Chicory pellets and powder	OTA	Stationary	15	120–357	<0.16	—	<0.01–1.1	<0.03	—	—
		Aflatoxins	Stationary	2	120–166	1.2	—	0.99–1.34	1.95	—	1.4–2.5
IV	Blends of spices	OTA	Stationary	4	250–355	14.9	10.1	4–35	0.15	0.08	0.01–0.4
		OTA	Personal	10	35–331	53.4	28.2	10–167	0.85	—	<0.03–5.24
V (Day 1)	Blends of spices	OTA	Stationary	4	184–222	18.6	17.9	15.5–23.2	0.75	0.79	0.43–1.008
V (Day 2)				3	255–336	8.4	4.8	4.5–15.9	0.39	0.304	0.100–0.765
V (Day 3)				3	181–202	1.6	1.5	1.19–2.07	0.048	0.043	0.04–0.060
V (Day 1)		OTA	Personal	2	159–199	52.9	—	36.6–69.2	1.2	—	0.38–1.99
V (Day 2)				2	254–336	23.1	—	14.9–31.4	1.4	—	0.82–1.9
V (Day 3)				2	205–285	14.4	—	9.5–19.4	0.26	—	0.03–0.49
V (Day 2)		Aflatoxins	Stationary	1	249	3.6	—	—	5.3	—	—
V (Day 3)				1	283	26.5	—	—	10.6	—	—

contamination of the bulk material. This confirmed the comparisons experimentally made between the raw matrix contamination and the aerosol particle contamination. Recently, S. Biselli *et al.* have also presented data establishing the fact that mycotoxins accumulate on dust particles, sometimes up to 35-fold in comparison to contents in ground bulk grain.[53] The measurements were used to assess the likely occurrence of airborne mycotoxins, and were not legal exposure measurements. They were considered as a field validation intended to highlight any possible difficulties linked to mycotoxin air sampling compared to real-life occupational monitoring activity. Even though there was no occupational or environmental limit value to be compared to, the results provided relevant information. They confirmed that workers may be exposed to mycotoxins when handling contaminated cereals, root vegetables and spices. Until now, no results have been published for fumonisin air measurements. OTA was detected and measured both in the foodstuffs and in the air inhaled by the workers for all the activities investigated, with the exception of site (III). In fact, chicory OTA contamination has never before been published. The levels measured lay within the proposed application range of the methods. They

were also high enough to demonstrate that the CIP-10 personal sampler was fit for purpose, with its collection capacity fully adapted to the concentration of particulate matter. A few aflatoxin measurements were taken, but the substances were detected on all the samples. The presence of this type of mycotoxin is quite worrying, even though a causal relationship between air exposure and human disease has not yet been established. The so-called aerosol contamination was estimated for each air sample. It could not be directly compared to the foodstuff contamination as it was not possible to analyze all the blends or bulk materials handled by the workers. For the most part, large bag unloading was a particularly dusty job. Although short-term peak levels were measured, they could have occurred again during the day, leading to significant or high mycotoxin exposure. This would explain, while not having justified it, why additional respiratory protective equipment such as a face mask or a respirator should be worn. If the masks fitted tightly against the face and provided protection against harmful dusts, they could reduce and even eliminate exposure in this case.

Regarding the rare previously published data, the reported limits of quantification could not always be directly compared with ours.[16,20–22] They were sometimes calculated from the analytical limit of detection linked to the highest air volume imaginable. This, in our opinion, is a much too optimistic way of proceeding. The sampling yield is usually far less than 100%, as is the analytical treatment of the sample. Some published values seemed incorrectly reported in ng m^{-3}. Once they are corrected, far higher airborne aflatoxin concentrations appear to have been detected, but obviously in very particular situations, such as non-detoxified soybean unloading or poultry houses. The ochratoxin A levels measured by other authors in the past, when detected, were of the same order of magnitude.

Conclusion

Methods to monitor occupational exposure to seven frequently occurring airborne mycotoxins have been described and optimized. They have been successfully validated and meet the criteria required of reproducible and reliable methods for personal workplace sampling. The measuring procedure has been designed to directly measure a minimum air concentration of 0.015 ng m^{-3} OTA, 1 ng m^{-3} fumonisins or 0.5 pg m^{-3} aflatoxins, with a 4800 L air volume sampling in a wide range of dusty environments. The results of the experimental controlled exposure studies were compiled to validate the methods and to show how well they performed. Contrary to other studies focussing on the measurement of mycotoxins in air or in food samples, the present study was not solely based on liquid spiking where the mycotoxins are probably not as strongly bound as in dust particles. This results in a more representative sampling procedure and sample analysis. Several types of mycotoxin and very different contamination levels can pose relative difficulties for laboratories not participating in food proficiency testing. Thus, a specific air sampling strategy and a very detailed analytical protocol have been proposed in the *Metropol* database, which is available on the INRS website for occupational operator awareness raising purposes. Measurements using conventional analytical equipment can now be performed. The user-friendliness of our methods will be further improved, if this is possible, by testing the use of a less expensive extraction device for analyzing OTA. We are currently evaluating commercially available molecularly imprinted polymer cartridges to check whether these complementary analytical tools could allow better analytical sensitivity.[54]

The methods were used for a number of field measurements when workers handled contaminated cereals, root vegetables or spices. Significant air levels were detected whereas the food contamination was below the regulatory limits. Some authors had assumed that the proportion of mycotoxin in raw material, settled dust and airborne dust was similar, but this may have given biased estimates.[55–57] Mycotoxins are indeed dangerous substances, and are of sufficient concern to justify the assessment of occupational exposure. More recent publications confirm that new occupational measurements, where raw or bulk material contamination is possible, could be relevant in assessing the need for future research on the action mechanisms and subsequent occupational risk exposure limits setting.[56–61]

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