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Ultra-sensitive, stable isotope assisted quantification of multiple urinary mycotoxin exposure biomarkers

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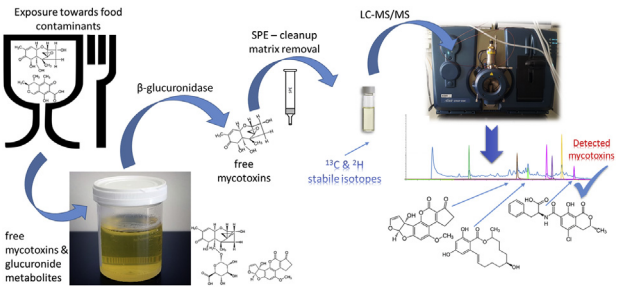
HIGHLIGHTS

- An ultra-sensitive method for urinary biomarkers of mycotoxin exposure was established.
- Simultaneous biomonitoring of regulated and emerging mycotoxins at trace levels by a single analytical method.
- First multiple stable isotope assisted quantification method for mycotoxin exposure biomarkers validated.
- Applicability in realistic chronic low dose exposure to mycotoxins in large-scale cohort.

GRAPHICAL ABSTRACT



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ABSTRACT

There is a critical need to better understand the patterns, levels and combinatory effects of exposures we are facing through our diet and environment. Mycotoxin mixtures are of particular concern due to chronic low dose exposures caused by naturally contaminated food. To facilitate new insights into their role in chronic disease, mycotoxins and their metabolites are quantified in bio-fluids as biomarkers of exposure. Here, we describe a highly sensitive urinary assay based on ultra-high performance liquid chromatography - tandem mass spectrometer (UHPLC-MS/MS) and ^{13}C-labelled or deuterated internal standards covering the most relevant regulated and emerging mycotoxins. Utilizing enzymatic pre-treatment, solid phase extraction and UHPLC separation, the sensitivity of the method was significantly higher (10-160x lower LODs) than in a previously described method used for comparison purpose, and stable isotopes provided compensation for challenging matrix effects. This method was in-house validated and applied to re-assess mycotoxin exposure in urine samples obtained from Nigerian children, adolescent and adults, naturally exposed through their regular diet. Owing to the methods high sensitivity, biomarkers were detected in all samples. The mycoestrogen zearalenone was the most frequently detected contaminant (82%) but also ochratoxin A (76%), aflatoxin M_1 (73%) and fumonisin B_1 (71%) were quantified in a large share of urines. Overall, 57% of 120 urines were contaminated with both,

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Liquid chromatography tandem mass spectrometry

aflatoxin M_1 and fumonisin B_1, and other co-exposures were frequent. These results clearly demonstrate the advanced performance of the method to assess lowest background exposures (pg mL^{-1} range) using a single, highly robust assay that will allow for the systematic investigation of low dose effects on human health.

1. Introduction

Characterizing complex environmental exposures and their combined effects on toxicity and human health has become a priority recently and is often referred to in the context of the 'exposome' paradigm [1–3]. Mycotoxins are a major class of natural contaminants that humans are typically exposed to throughout their life. This chemically diverse group of toxic secondary metabolites are produced by filamentous fungi and frequently occur in our diet [4]. The patterns and concentrations largely depend on climatic conditions as well as the level of hygienic standards and economic wealth. Higher mycotoxin exposures are often evident in tropical and sub-tropical regions of third world countries [5,6], although the changing climate is also altering occurrence patterns and concentrations throughout the world [7]. The toxicities of several mycotoxins have necessitated their regulation in almost all countries world-wide [8], though less affluent regions often lack the capacity to effectively implement or control such guidelines to protect their population. The permitted concentrations of major mycotoxins in the European Union are regulated within EC 1881/2006 [9]. For some mycotoxins including the carcinogenic aflatoxins, permitted levels are typically very low(e.g. aflatoxin M_1 (AFM_1) in milk: 50 pg mL^{-1}). However, it is known from biomarker driven research that combined exposures to mycotoxins are common [10–13]. Moreover, there is growing scientific evidence that mixtures of co-occurring mycotoxins [14–16] and mycotoxins with other xenobiotics (bioactive food constituents, drugs etc.) [17,18] have the potential to cause an additional threat through combinatory effects that legislation does not take into account to date.

To assess multiple exposures to mycotoxins, LC-MS/MS-based methods have been successfully developed to measure the parent compound or their metabolite(s) in urine, and tested mostly in smaller pilot surveys [10,12,13,19]. However, typically they constitute a compromise on assay sensitivity compared to single-analyte methods utilizing tailored sample clean-up protocols. Published multi-mycotoxin LC-MS/MS methods include rapid and cost-effective but partially less sensitive dilute and shoot approaches [20,21], a (semi)-quantitative direct injection method [19], sample clean-up using highly specific but expensive immunoaffinity (IAC) columns [19,22] and the combination of IAC columns with solid phase extraction (SPE) columns [13] which is time- and cost intensive. Moreover, salting out assisted liquid/liquid extraction [23–25], and a combination of liquid/liquid extraction with SPE columns [26] was described. Urine is used as a non-invasive, easily obtainable material for estimation of exposure to mycotoxins. Since humans are exposed to mycotoxins mainly through diet (with some exceptions where they can be exposed also through dust [27]), the main absorption region is the small intestine and transfer to the liver [28,29]. Via their normal diet, humans are also exposed to modified ("masked") mycotoxins, which may have different adsorption patterns. The typical conjugation to sugars (mainly glucose) is making them more resistant to adsorption in the small intestine. When reaching the colon, the microbiota can hydrolyse the conjugated form [30,31], and the "parent" mycotoxin is released and may be adsorbed there, following the same route to liver by portal vein, and metabolism process. Modified mycotoxins are an issue since they are not regulated to date and not covered by most analytical methods, although they can significantly contribute to the overall mycotoxin exposure [30–32]. For some mycotoxins such as deoxynivalenol (DON), this process is highly efficient and glucuronides are the major metabolites found in urine [33]. Therefore, the measurement of parent toxins often resulted in insufficient correlations with dietary intake estimates and the direct assessment of conjugated forms [34] or pre-treatment with β-glucuronidase/arylsulfatase was suggested [35–37].

From an analytical perspective, urine is known as a challenging matrix particularly due to vast differences in composition and concentrations between individuals, which may depend on sex, age, health status, metabolism and predominantly diet [10]. For mycotoxin biomarkers urine, blood (plasma/serum), milk, and hair may be used, depending on the targeted mycotoxin, exposure timeframe, or available analytical technique [38]. Urine was used most often in the past to describe recent exposure [38], since most of mycotoxins are rapidly metabolised and excreted via urine [33]. Sample pre-treatment with beta-glucuronidase is important to reconvert conjugates of mycotoxins back to parent mycotoxins. There is limited availability of mycotoxin glucuronide standards on the market and as they are mostly synthetized in small quantities in-house, hence de-glucuronidation can help in analysing total exposures if reference standards are not available or too costly. However, for estimating total exposure both, the direct and the indirect assessment of metabolites proved their feasibility [34,39]. To effectively compensate for varying matrix effects, extraction losses and other potential issues during LC-MS/MS quantification of mycotoxins, stable isotope dilution assays are often used as the state-of-the-art technique for data quality assurance [40]. Despite its advantages, no stable isotope labelling (SIL) workflow was reported for multi-mycotoxin exposure assessment in human biofluids to date to the best of our knowledge.

Hence, the aim of this study was to develop a highly specific and robust method for multi-mycotoxin biomarker analysis using tailored sample clean-up and stable isotopologues. Its feasibility and performance to assess individual exposure levels was demonstrated in a well-defined sample collection with urines reflecting a wide range and diverse mix of mycotoxins, a set likely to cover typical ranges in large-scale epidemiological studies.

2. Materials and methods

2.1. Reagents and chemicals

Methanol (MeOH; LC gradient grade), acetonitrile (ACN; LC gradient grade), and glacial acetic acid (HAc; MS grade) were purchased from Merck (Darmstadt, Germany). Mycotoxin standards were purchased from Romer Labs Diagnostic GmbH Tulln, Austria: nivalenol (NIV), ^{13}C-NIV, deoxynivalenol (DON), ^{13}C-DON, deepoxy-DON (DOM-1), ochratoxin A (OTA), ^{13}C-OTA, aflatoxin M_1 (AFM_1), ^{13}C- AFM_1, citrinin (CIT), fumonisin B_1 (FB_1), ^{13}C-FB_1, ^{13}C-zearalenone (ZEN)) or Sigma, Vienna, Austria (ZEN, α- and β-zearalenol (ZEL)). The deuterated [$^2H_4$] alternariol (AOH) was synthesized in-