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Review

Measuring Human Mycotoxins Exposure Using Biomarkers – A Review

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Abstract: Mycotoxins are secondary toxic compounds produced by fungi of the Aspergillus, Fusarium and Penicillium species during secondary metabolism and they pose a major threat to humans and animals. Estimation of mycotoxin exposure by measuring contamination of food coupled with dietary intake questionnaires has been improved by the availability of biomarkers of exposure, which allow more accurate and objective exposure measurements. Biomarker assessment in biological fluids has greatly contributed to elucidating the mechanism of health impairments attributable to these toxic compounds and to studying their pharmacokinetics. The analytical methods for the determination of single mycotoxin exposure biomarkers include enzyme linked immunosorbent assay (ELISA), high performance liquid chromatography (HPLC)-fluorescent detection and Gas chromatography (GC)-electron capture, and HPLC mass spectrometry. Recent advances in liquid chromatography tandem mass spectrometry (LC-MS/MS) have revolutionised mycotoxin biomarker assessment through the analysis of multiple mycotoxins in one sample injection. Despite these current developments, efforts still need to be made towards further improvements in sampling procedures, inter-laboratory quality assurance and development of internal standards to ensure appropriate detection of biomarkers.

Keywords: Mycotoxins; Biomarkers; Biological samples; Enzyme linked immunosorbent assay; Liquid chromatography tandem mass spectrometry.

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1. Introduction

Mycotoxins are secondary metabolites of fungi, especially *Aspergillus*, *Fusarium* and *Penicillium* species contaminating 25% of food crops worldwide and therefore, are regarded as major health threats to humans and animals[1]. Aflatoxins, fumonisins, ochratoxins, deoxynivalenol and zearalenone are regarded the most worldwide concerned mycotoxins due to their frequent occurrences in cereals and cereal-based products. In evaluating the risk associated with a toxicant, biomarkers are used to provide data on contamination-related responses in a biological system or quantify the toxicant and/or its biotransformation metabolites in biological fluids. According to World Health Organisation (WHO) '*biomarker is used in a broad sense to include almost any measurement reflecting an interaction between a*

biological system and an environmental agent, which may be chemical, physical or biological' [2]. Three classes of biomarkers include biomarkers of exposure, biomarker of effect and biomarker of susceptibility. WHO[2] also defined biomarker of exposure '*as an exogenous substance or its metabolite or the product of an interaction between a xenobiotic agent and some target molecule or cell that is measured in a compartment within an organism*.' Biomarkers of exposure are indispensable tools in the assessment of the health effects associated with mycotoxins in humans and animals as well as monitor the outcome of intervention studies geared towards mycotoxins exposure reduction. This review examines the different biomarkers of exposure for aflatoxins, ochratoxin A, fumonisins, zearalenone and deoxynivalenol and their current detection methods.

2. Single-mycotoxin biomarker methods

2.1 Ochratoxin A

Ochratoxins are mainly produced by *Aspergillus* and *Penicillium* species and contaminate cereals, coffee, dried fruit and other products. Ochratoxins include Ochratoxin A (OTA), its methyl ester, its ethyl ester (Ochratoxin C), 4-hydroxyochratoxin A, Ochratoxin B with its methyl and ethyl esters and Ochratoxin α (**Figure 1**). OTA is the most toxic of all the ochratoxins and studies shows that OTA is nephrotoxic, hepatotoxic, embryotoxic, neurotoxic, immunotoxic, genotoxic and carcinogenic, and have been classified as a possible human carcinogen (group 2B)[3]. Health risk assessment of exposure to OTA and other mycotoxins were previously done by measuring their concentrations in foodstuff combined with data of food consumption and dietary habits but this failed to provide accurate data on human exposure[4].

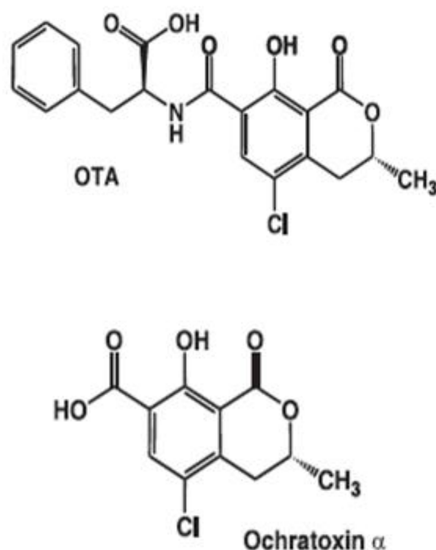

Fig. 1: Chemical structure of ochratoxin A (OTA) and ochratoxin α

However, with the availability of validated exposure biomarkers, the assessment of dietary exposure of humans to mycotoxins, including OTA are done by estimating their level in biological samples using biomarkers of exposure[4]. Exposure assessment using biomarkers is an indispensable tool due to its ability to provide information on mycotoxins

exposure by all routes (oral, dermal, inhalation), and in situations where food contamination data are scarce or not available[5),6)]. According to Scott[5)], OTA binds strongly to serum albumin and/or other proteins allowing OTA to possess a long serum half-life ($t_{1/2}$ 35 days) and therefore, detection of OTA in blood serum is an important biomarker for human OTA exposure. In addition, urinary OTA is considered to be a human exposure biomarker[7)]. The OTA concentrations in blood serum/plasma gives data for exposure over longer periods, while biomarker analysis in urine only gives information on day-to-day variations in exposure of adults and infants[4),6),8)].

Several analytical methods have been employed in the detection of OTA exposure biomarkers in humans. Castegnaro et al.[9)] used an HPLC-flourescence detection (HPLC-FD) method to analyse OTA in serum and urine of villagers from a region of Bulgaria with high prevalence of Balkan Endemic Nephropathy but found that the biomarkers were not directly correlated with weekly OTA intake based on OTA consumption estimated by food intake/contamination analysis[9)]. More recently, Ali et al. have used the HPLC-FD method to measure OTA exposure using serum or urine samples in Bangladeshi populations, showing frequent exposure to OTA[6),10)].

In a pilot study using LC-MS/MS, OTA and OTα were simultaneously detected in the plasma and urine of 13 volunteers[11)]. It was observed that OTA was the predominant form found in plasma while the OTα metabolite was mainly found in the urine. Concentrations of OTα in urine (mean: 2.88 ± 2.24 ng/mL) were higher than OTα concentrations in plasma (mean: 0.95 ± 0.46 ng/mL). In addition, urinary OTA (mean: 0.07 ± 0.05 ng/mL) was lower than the value found in plasma (mean: 0.25 ± 0.03 ng/mL) of the subjects. In a study in Spain, 72 urine samples were treated enzymatically with glucuronidase/arylsulfatase, separated with liquid extraction and examined with high performance liquid chromatography-fluorescence detection (HPLC-FLD)[12)]. OTA was detected in 9 out of 72 samples (12.5%; limit of detection, 0.034 ng/mL) and OTα in 44 samples (61.1%; limit of detection, 0.023 ng/mL). Furthermore, the concentrations of OTA and OTα were 0.057-0.562ng/mL and 0.056-2.894ng/mL, respectively. They also observed that food frequency questionnaire and three-day food intake record was related to the concentration of OTA and OTα in urine, respectively. Similarly, OTA and OTα have been found in 40 urine samples from pregnant women using HPLC-FLD after liquid-liquid extraction using 1% NaHCO3 and chloroform/ispopropanol mixture (97:3, v/v) [13)]. In their study, part of the samples were first extracted with liquid-liquid extraction and another part extracted after enzymatic treatments. OTA was found in 75% and 58% of the samples before and after enzymatic treatment, respectively. It was also observed that the levels of OTα increased significantly in the samples enzymatically treated.

A comparison of enzyme-linked immunosorbent assay (ELISA) and HPLC-FLD in the detection of OTA in 115 serum samples was conducted by Dohnal et al.[14)]. The LOD of HPLC-FLD and ELISA was similar at 0.04 µg/L and 0.05 µg/L, respectively, with recoveries of 83 to 86% (HPLC-FLD) and close to 100% (ELISA), and there was linear correlation between both methods (r = 0.907). ELISA showed cross-reactivity of OTA antibody with other ochratoxins; ochratoxin B (OTB), Ochratoxin C (OTC) and ochratoxin α (OTα), and the presence of phenylalanine moiety in OTA molecule, as there is possibility of free phenylalanine and protein-bound phenylalanine competing with OTA antibody in ELISA[14)]. ELISA was also used by Erkekog˘lu et al.[15)] to assess the seasonal variation in serum ochratoxin A and 98% of the blood samples collected in summer had detectable level of OTA (0.028-1.496 ng/mL) whereas 76.1% in samples collected in winter showed OTA level in the range 0.031-0.887 ng/mL.

Immunoaffinity cleanup and HPLC-FLD have also been applied in the quantitation of OTA in plasma[16),17)] and urine[7),18),19)]. Pena and colleagues[18)] detected OTA in urine samples by extraction with 5% NaHCO3, immunoaffinity

column for cleanup and HPLC-FLD. The LOD was 0.02 ng/mL of urine with OTA recovery above 90% with relative standard deviations (RSD) of less than 9% demonstrating that the method has high accuracy and precision. Similarly, Akdemir et al.[7] showed recovery values of OTA in urine sample to be between 85.7% and 110.5% (limit of quantification: 0.006 and 0.018 ng/mL) and RSD values between 3.83 and 8.86 % (spiked concentrations: 0.02 to 1 ng/mL). This is true as the use of immunoaffinity chromatography and HPLC-FLD has been shown to increase the selectivity and sensitivity of the method[20]. However, immunoaffinity columns are only specific to OTA and there is a possibility of losing OTα as it is not retained[12].

Exposure to OTA varies significantly between climatic conditions, dietary habits and geographical location, and in several studies, there has been poor correlation between dietary intake and serum or urine OTA concentrations. However, many studies have applied the Klassen equation[5),12),15)] to relate dietary intake of OTA with plasma concentrations, plasma clearance (renal filtration) and bioavailability (k_o = 0.67 x C_p/0.5 = 1.34 x C_p; where k_o = continuous dietary intake (ng/kg body weight/day), 0.67 = renal clearance rate (mL/kg body weight/day), 0.5 = bioavailability of OTA (fraction of OTA taken up) and C_p = plasma concentration (ng/mL).

2.2 Deoxynivalenol

Deoxynivalenol (DON) is one of the type B trichothecenes which are toxic sesquiterpenoid mycotoxins produced by *Fusarium*, *Stachybotrys* and *Myrothecium species* of fungi during their growth in food and/or the environment (**Figure 2**). Different methods have been applied in the analysis of deoxynivalenol (DON) in urine, including gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) after purification with immunoaffinity column (IAC), solid phase extraction (SPE), or conjugation of both IAC and SPE[21].

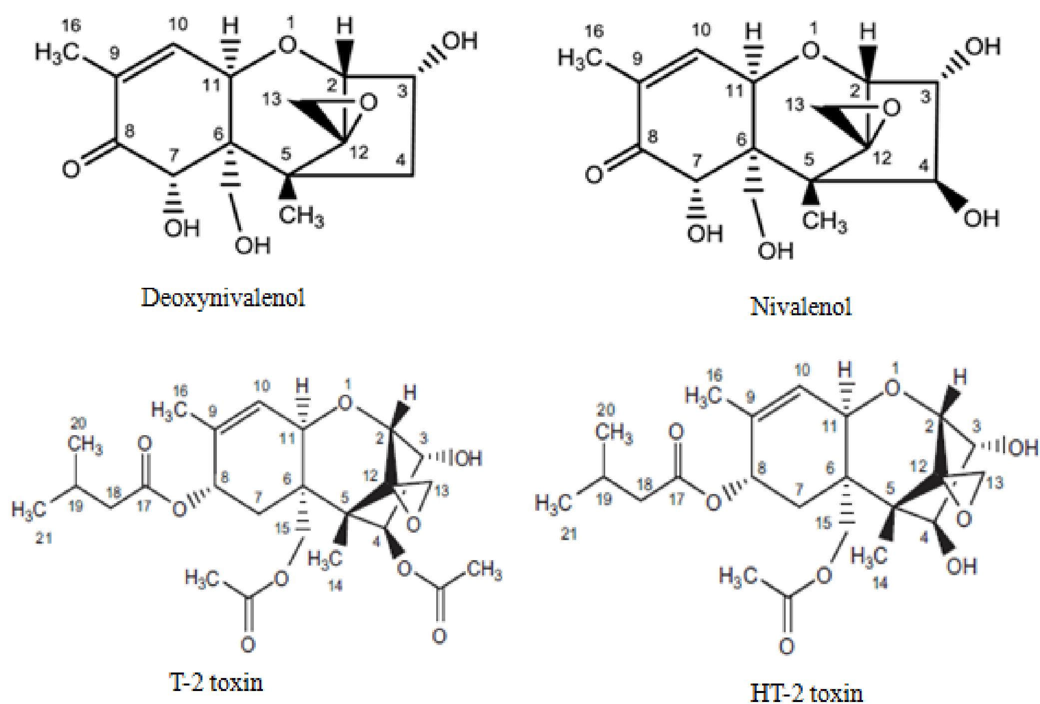

Fig. 2: Chemical structures of major trichothecenes

In 2003, Meky and colleagues pioneered the assessment of deoxynivalenol (DON) in urine using immunoaffinity enrichment and liquid chromatography-mass spectrometry[22]. This method was improved on by using $^{13}C_{15}$-DON as an internal standard following an optimised enzymic digest[23] and this has revolutionalised the urinary assessment of DON. The method had a limit of quantification (LOQ) of 0.6 ng DON/mL urine with coefficient of variation of 6.1% and was used to assess DON exposure by measuring urinary DON levels in 25 UK volunteers. This method was applied in both DON exposure measurement and intervention studies on DON urinary levels. In the days following the intervention procedure, volunteers took normal diets (composed mainly of wheat products) and all 25 volunteers had urinary DON while only 9 of the 25 had detectable urinary DON during the intervention period. In addition, the geometric mean of urinary DON in period of normal diet (7.2 ng DON/mg creatinine; 95% CI 4.9-10.5 ng/mg) was 11-fold significantly higher than that found during the intervention process (0.6 ng/mg; 95% CI 0.4-0.9 ng/mg) indicating that intake of wheat-based foods such as bread and pasta is associated with urinary DON levels. The reduction of urinary DON in the 4-day intervention could be because of rapid excretion of the previously ingested DON; studies in rats indicate that ingested DON can be eliminated within 4 days[22] and the non-detection of conjugated DON could be due to treating the urine samples with β-glucuronidase causing liberation of DON from conjugates. In another study in UK, DON was detected in 296 of 300 (98.7%) urine samples (geometric mean: 9.42 μg DON/day; range: undetectable - 65.97 μg/day) using LC-MS after purification on immunoaffinity columns[24]. Total urinary DON (fD + DG) concentration was significantly associated with cereal consumption ($p < 0.005$). Similarly, total urinary DON showed significant association with cereal consumption both as a continuous variable ($p < 0.005$) and as a categorical variable ($p < 0.005$), even after creatinine adjustment[24]. Wheat-based meals were also associated with urinary DON levels like that previously reported[23] indicating that wheat-based meals are the most common source of DON exposure in UK.

In a different study, Cunha and Fernandes[21] compared the application of immunoaffinity column (IAC) and solid-phase extraction (SPE) in combination with gas chromatography-mass spectrometry (GC-MS) in the detection of deoxynivalenol and metabolites in human urine. This was the first report of the application of SPE in the extraction of DON and its metabolites followed by GC-MS analysis. In addition, they compared the effectiveness of three different β-glucuronidase enzymes (type IX from *E. coli*, Type I from *H. pomatia* and type L II from *P. vulgate*) usually applied in the digestion of DON-glucuronide and metabolites from human urine. It was observed that type I enzyme from *H. pomatia* showed the best peak area followed by type IX from *E. coli* and type L II from *P. vulgate*[21]. Meky et al.[22] also reported high efficiency of type IX enzyme from *E. coli* in comparison with *P. vulgate*, but no comparison with type I enzyme from *H. pomatia* was done in their study. Furthermore, comparison of chromatograms obtained shows that the octadecylsilane (C18) SPE extraction cleanup had higher chromatographic signal for DON and metabolites. Using this technique, the levels of detection and quantification ranged from 0.06 - 0.30 ng/mL and 0.2 - 1.0 ng/mL of urine, respectively. The recoveries were between 73% and 105% for fortification levels between 25 and 100 ng/mL with repeatability values ranging from 5% to 13%. The repeatability values obtained were like that previously reported[23,25,26,27] while the levels of quantification was similar to that by Turner et al. [23,25] with 0.5 ng/mL urine for DON and 0.06 ng/mL for DOM-1, and by Solfrizzo et al. [26] with 0.8 ng/mL for both DON and DOM-1. However, the use of IAC cleanup and LC-MS/MS is superseeded by this method developed by Cunha and Fernandes[21] as the cost of sample preparation was reduced by using C18 SPE cleanup in combination with low volumes of extraction solvent.

Reports show un-metabolised DON and conjugated DON as the main compounds detected in human urine[23] and therefore, simultaneous measurement of free DON (fD) and DON-glucuronide (fD +DG) in urine has been recognised as a human exposure biomarker for DON intake[23],[25]. Urinary fD + DG levels have been correlated with cereal intake[24] and the level of fD +DG combined has shown significant correlation with urinary free DON[28]. This is highly corroborated by a recent study in Tanzania conducted by Srey and colleagues[29] in which DON intake in children was well correlated with urinary DON. In this study, IAC was used in combination with LC-MS-ESI and the LOD was 0.5 ng DON/mL of urine. The geometric mean for DON level was 2.5 ng/mL of urine with urinary DON and fDON were found in 51% and 22 % of the urine samples from children from Tanzania aged 6-14 months, respectively. In Tanzania, correlation between DON urinary biomarkers was seen in children and mothers but in men, additional intake of DON from local maize brew was associated with the urinary DON biomarker[30]. The total DON (free + conjugated) with levels ranging from 0.5 - 28.8 ng/mL was found in urine samples from France[31], 5.0 - 78.2 ng/mL in UK[28] and 67% of urine samples (range: 1.9 - 26.2 ng/mL; mean: 16.3 ng/mL) in Portugal[21] while free DON in urine was found in 23 of 34 urine samples (0.5 to 9.3 ng/mL) in UK[28] and 2 of 13 (15%) subjects (1.8 and 8.8 ng/mL) in Portugal[21]. Although correlations between the urinary levels of DON and DON-glucuronide and dietary intakes of DON have been recorded, there is need to apply inter-laboratory surveys in order to validate their use as biomarkers of DON exposure.

De-epoxy metabolite of DON (de-epoxy-deoxynivalenol; DOM-1) has also been found in 26 of 76 (34%) urine samples from individuals exposed to DON in Normandy, France[31] and 2% of urine samples examined in UK[28]. In contrast, Turner et al[32] did not detect DOM-1 in their biomarker survey of urinary deoxynivalenol in 60 women from Shanghai, China but DON-glucuronide was found in 96.7% (mean 4.8ng DON/ml urine or 5.9ng DON/mg creatinine) of the samples using HPLC-MS. DOM-1 was not detected in urine samples of UK adults[25] and all 13 urine samples examined in a pilot study in Portugal[21]. In the French survey by Turner and colleagues, DOM-1 were mainly found in the urine samples of farmers handling cattle suggesting acquisition of animal microbiota with de-epoxidase action as DON is metabolised to DOM-1 in the rumen of cows[31]. The inconsistent detection of DOM-1 in human urine is an indication that it is possibly not a major DON metabolite in humans and therefore, may not be important exposure biomarker in the biomonitoring of human disease risk associated with DON. Interestingly, Warth et al.[33] developed a LC-MS/MS method of detecting DON and DON-3-glucuronide in human urine. This method bypasses the enzymatic digestion of DON-glucuronides and uses an internal DON-3-glucuronide standard, which allows the use of 'dilute and shoot' method removing the rigorous cleanup step. The limit of detection of DON-3-glucuronide was 3 μg/L with recovery of approximately 88% whereas LOD and recovery for DON was 6 μg/L and 101% respectively. Similarly, Sarkanji et al.[34] detected DON-3-glucuronide and DON-15-glucuronide in 97.7% of urine samples of pregnant women from Croatia using Liquid chromatography-electrospray ionisation tandem mass spectrometry (LC-ESI-MS/MS) and a 'dilute and shoot' method. These indicate the great prospects of using DON-3-glucuronide and DON-15-glucuronide as biomarkers of human DON exposure. However, the DON-3-glucuronide standard employed in these tests is not commercially available and its availability will be of great potential in the direct quantification of DON and its glucuronide metabolites. This will be beneficial in the validation of DON biomarkers for application in biomonitoring of human exposure to DON and intervention programmes.

2.3 Aflatoxins

Aflatoxins are secondary metabolites of *Aspergillus flavus* and *A. parasiticus* and are one of the major contaminants of peanuts, cottonseed, corn and rice (**Figure 3**). The naturally occurring classes of aflatoxins are AFB1, AFB2, AFM1, AFM2, AFG1 and AFG2, and their hydroxylated metabolites commonly found in milk and urine (AFM1, AFM2)[35)]. Aflatoxin B1 is the most toxic, causing acute liver toxicity that can lead to death after high exposure[36),37)], and hepatocellular carcinoma, particularly in individuals with chronic hepatitis B virus infection[3)], after chronic exposure. AFB1 has also been implicated in growth impairment in children[38),39),40),41)] and modification of immune function[42)]. The development of validated biomarkers and analytical techniques has improved research into the influence of aflatoxins on human health[43),44)]. By the late 1980's the potential of the urinary DNA adducts, as a short-term biomarker of exposure[45),46),47)] and the serum albumin adduct as a measure of accumulated exposure over 2-3 months[48),49)] had been established. Both biomarkers were further validated in studies on human populations in high exposure regions of China and Africa[50),51),52),53)]. In addition, urinary aflatoxin M1 (AFM1) has been shown to have dose-dependent association with dietary consumption of AFB1 contaminated foods and therefore, used as a biomarker for short-term exposure to aflatoxins[50),54),55),56),57)]. A key study that established the value of such biomarkers was the demonstration that the risk of liver cancer in a cohort of men from Shanghai region of China was enhanced in men who had tested positive for both aflatoxin exposure and hepatitis B virus infection[58)]. This study used the urinary aflatoxin DNA adduct to determine aflatoxin exposure and without the use of biomarkers to confirm exposure this study would not have been possible.

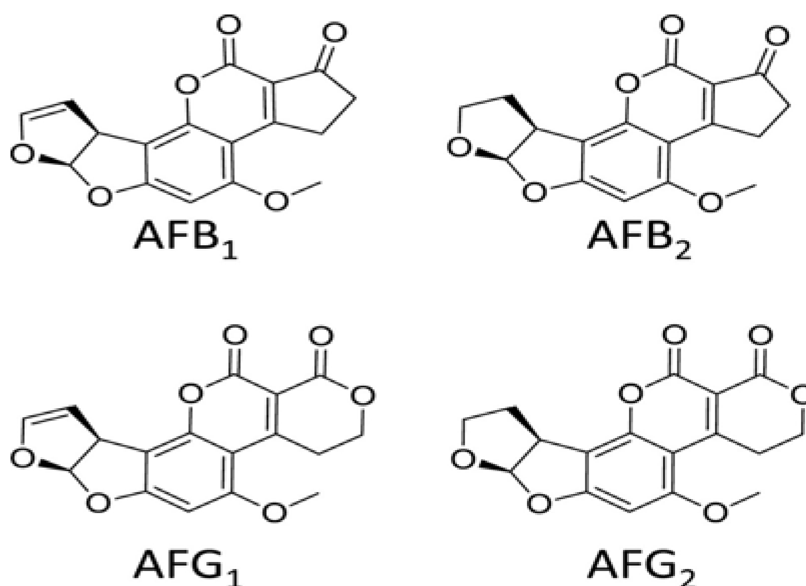

Fig. 3: Chemical structures of the major aflatoxins found in food

The dietary intake of aflatoxins has been reported to show significant association with the level of AFB1-albumin adduct in plasma samples[53),59)] and this led to its wide application as biomarker of human aflatoxins exposure. Two approaches to measuring AFB1-albumin have generally been taken- using an ELISA method to measure adduct levels[60)] or an HPLC method with either fluorescence or mass spectrometry to quantify aflatoxin B1-lysine adducts[61),62),63)]. Over the last decade highly prevalent exposure to aflatoxin has been reported using the ELISA method for detecting serum AFB1-albumin adducts in many African countries including Tanzania, Kenya, Gambia, Senegal, and

Guinea and Uganda[64),65),67),68),69)]. Levels of AFB1-albumin were found to correlate with dietary intake of aflatoxin in Tanzanian children[70)]. Mean values of AFB1-alb adduct ranged from 10 pg/mg albumin in Ugandan children to 540 pg/mg in Kenyan adolescents. Amongst this body of research, the highest levels of biomarker were recorded in Kenya in 2004 during a period of high aflatoxin contamination which resulted in acute aflatoxicosis related deaths in parts of the country[66)]. These studies have shown that aflatoxin exposure can vary with season, year on year and geographically, including due to local microclimate. Other laboratories have used chromatographic separation with fluorescence or mass spectrometry detection to measure AF-lys in populations from a number of countries. For example, Leong et al.[71)] applied HPLC-FLD to measure the serum AFB1-lysine adduct levels in 170 subjects from Malaysia. A total of 97% of the serum samples had AFB1-lysine adducts above the detection limit (0.4 pg/mg albumin) ranging from 0.20 to 23.16 pg/mg albumin and most of the subjects had levels ranging from 5.0 to 9.9 pg/mg albumin but there was no correlation between AFB1-lysine adduct level and dietary intake in the study population.

Reports show a significant correlation between different methods employed in the detection of serum/plasma AF-albumin (Af-alb) adduct, including ELISA and HPLC-FLD[72)], RIA and HPLC-FLD and ELISA and LC-MS/MS[61),71)], and HPLC-FLD and IDMS[62)]. However, immunoassay methods usually give higher level of AFB_1-albumin adduct than other methods, including HPLC-FLD, HPLC-IDMS and LC-MS/MS[60),61)]. For instance, McCoy et al.[63)] showed that there was high correlation between the concentration of aflatoxin-albumin adducts determined by ELISA and AFB_1-lysine determined by HPLC-FLD or IDMS in 2 mg of albumin. In contrast, AFB1-albumin adducts concentrations determined by ELISA were 2.6 fold higher than the level of AFB_1-lysine adducts probably due to incomplete enzymatic digestion, adduct formation with different amino acids, and presence of structurally related aflatoxins[63)]. Jolly et al.[55)] quantified serum AFB_1-albumin adducts and urinary AFM_1 using RIA and IAC-HPLC-FLD respectively and found a significant correlation between the levels of plasma AFB_1-albumin adduct and urinary AFM_1. A similar correlation has recently been reported between AFB_1-albumin measured by ELISA and urinary AFM_1 measured by ELISA in samples from Tanzania[73)].

2.4 Zearalenone and metabolites

Zearalenone (ZEN) produced by *Fusarium species* is also considered as one of the common mycotoxins posing a threat to human and animal health[74)]. ZEN and its metabolites (**Figure 4**), including α-zearalanol (α-ZOL), β-zearalanol (β-ZOL), α-zearalenol (α-ZAL), β-zearalenol (β-ZAL) and zearalanone (ZAN) are potent endocrine disruptors due to their resemblance to 17- β-oestradial enables them to bind to oestrogen receptor disrupting the binding of 17- β-oestradial. It has been reported to cause hyper-oestrogenism with attendant reproductive disorders and infertility problems in farm animals and humans[74)].

Different analytical methods have been applied in the measurement of zearalenone, including thin-layer chromatography (TLC), HPLC-FLD, ELISA, gas chromatography/mass spectrometry (GC/MS), liquid chramotography (LC) with carbon nanotube-modified electrode, liquid chramotography with UV diode array (HPLC-DAD), liquid chromatography/mass spectrometry (LC/MS) and liquid chromatography tandem mass spectrometry (LC-MS/MS)[75),76)]. Recently, LC/MS and LC/MS/MS have increasingly been applied for the detection of ZEN and its metabolites in biological fluids because these methods have been shown to determine their level in test samples with high sensitivity and selectivity regardless of the purity in biological substances[77)]. ZEN and metabolites have been determined concurrently in urine samples using LC/MS/MS assays[76),78),79),80),81),82)].

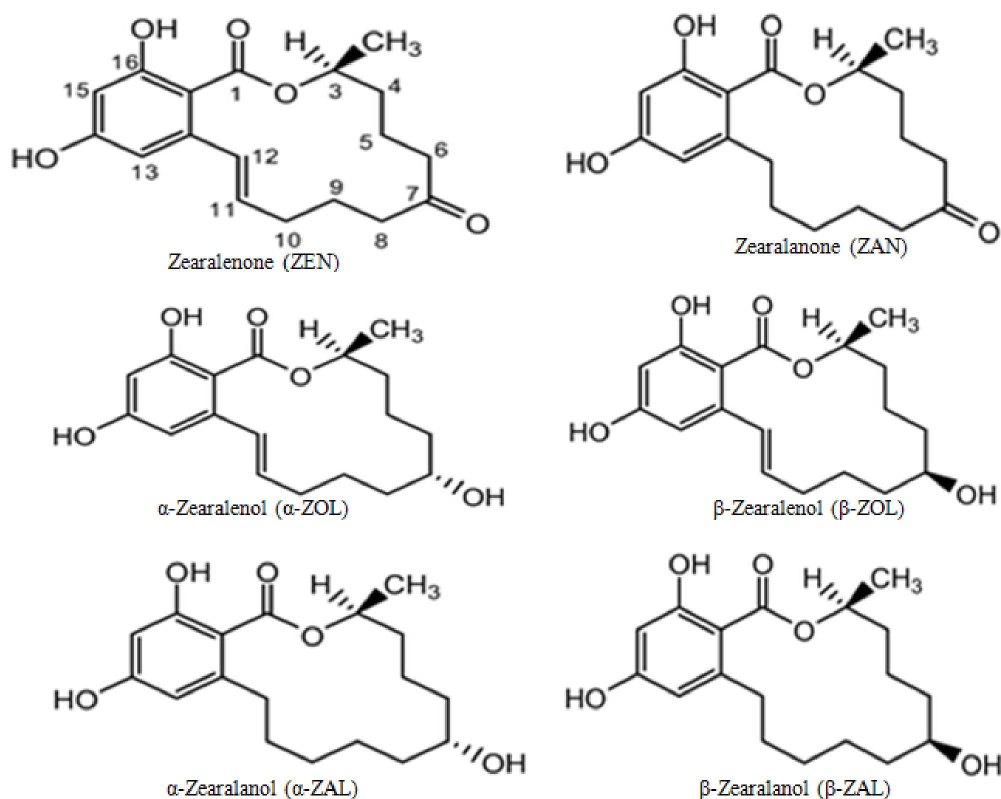

Fig. 4: Chemical structures of zearalenone and its major metabolites

The metabolism of ZEN in human beings is poorly understood. Therefore, most current information concerning the presence of ZEN in physiological fluids and compartments are available as pharmacokinetic studies in animal models[76),83)]. ZEN and its metabolites, including α-zearalanol (α-ZOL), β- zearalanol (β-ZOL), α-zearalenol (α-ZAL), β-zearalenol (β-ZAL) and zearalanone (ZAN) have been measured in urine (human, bovine and swine) using liquid chromatography with carbon nanotube-modified electrode[75)]. The method only allowed the detection of ZEN and metabolites in the range of 5 to 50 ng/mL (LOD: 5 ng/mL) and there was no correlation between the presence of ZEN and its metabolites in urine and its exposure. Recently, glucuronides of ZEN have been detected in human urine[81)]. Therefore, research should be focused on evaluating both ZEN and glucuronides in humans as possible biomarkers for ZEN. At present, it can be concluded that there is no validated biomarker of exposure for ZEN that could be used currently in assessing human exposure to ZEN.

2.5 Fumonisins

Fumonisins are mainly produced by *Fusarium verticillioides*, but can also be produced *F. proliferatum*, *F. anthophilum*, *Alternaria alternata,* etc[84)]. Currently, a total of 15 types of fumonisins, classified as A, B, C or P (**Figure 5**), are known with Fumonisin B_1 (FB_1), FB_2 and FB_3 being the most common forms with FB_1 the most toxic. FB_1 is the most widely studied due to its association with oesophageal cancer[85),86)] and neural tube defects[87),88)].

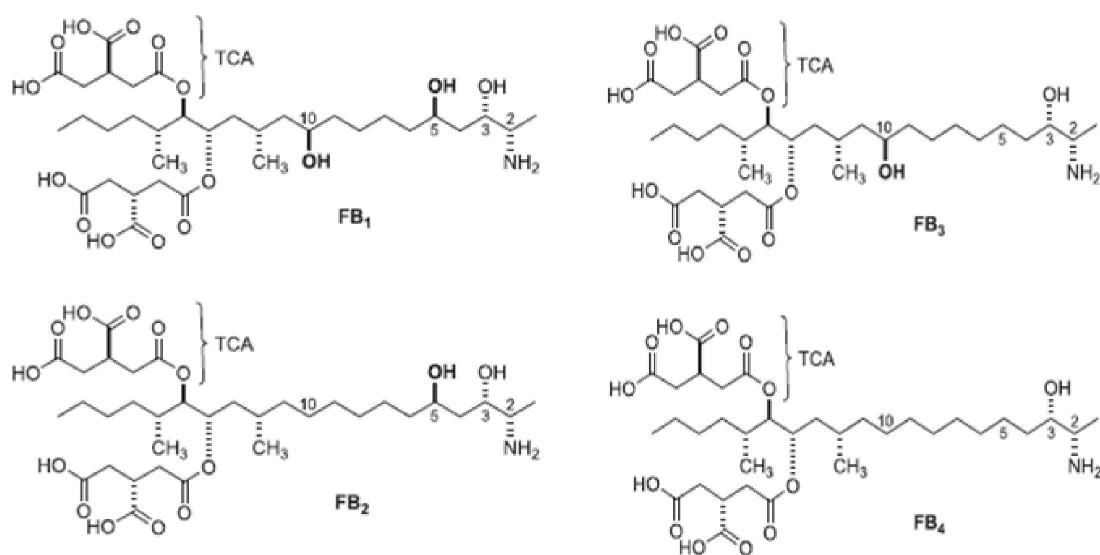

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Fig. 5: Chemical structures of fumonisins. 286

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The level of sphinganine-to-aphingosine ratio, elevated sphinganine level and the concentrations of FB_1 in hair and faecal samples were initially proposed as useful FB_1 biomarker in animals, but there was no correlation to human fumonisin exposure[89),90)]. Gong et al.[84)] determined FB_1 in urine samples of 75 women from Morelos, Mexico using LC-MS after extraction with Oasis MAX cartridges and reported a correlation of urinary FB_1 with tortilla consumption in the Mexican population. Urinary FB_1 represents only a small proportion of FB_1 intake but has been shown to be a useful biomarker of exposure. In a community-based intervention to reduce fumonisin intake from maize in rural South Africa, the reduction of urinary FB_1 was in good agreement with the reduction of dietary intake following the intervention[91)]. In Tanzania, levels of urinary FB_1 were found to vary geographically[64)], with good correlation between intake for mothers and children in a family study[30)], and an association between FB_1 exposure and impaired child growth in children under two years of age[92)]. Hence, this biomarker of FB1 exposure is proving valuable in monitoring exposure and possible health effects in areas of high exposure. 288–298

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3. Multi-mycotoxins biomarker detection 300

The advances in analytical techniques for the simultaneous determination of multiple mycotoxins biomarkers in grains- and biological samples enable assessment of multiple exposures to different mycotoxins in humans and animals (**Appendix A**). Ahn et al.[93)] pioneered the determination of multiple mycotoxin biomarkers in urine by analysing FB_1, FB_2, AFM_1, and OTA after concentration using three separate immunoaffinity columns. Since then, various analytical methods have been developed for multi-mycotoxin detection, including LC-MS/MS[27),81),82),94),95),96)], GC-MS/MS[97),98)], competitive fluorescent microsphere immunoassay (CFIA)[99)] and liquid chromatography heated electrospray ionisation tandem mass spectrometry (LC-h-ESI-MS/MS) [100)]. Mixtures of OTA, DON, AFM1, FB1, ZEN, α-ZOL and β-ZOL have also been quantified with LC-MS/MS in urine after liquid-liquid extraction (LLE) in combination with strong anion exchange (SAX) SPE clean-up with good recovery and sensitivity[101),102)]. In another study, Ezekiel et al.[103)] concurrently measured AFM1, DON, DON-15-O-glucuronide, FB1, OTA, FB2, ZEN, ZON-14-O-glucuronide in 301–310

urine samples from Northern Nigeria using 'rapid dilute and shoot' LC-MS/MS while. Solfrizzo et al.[26] reported developing LC-ESI-MS/MS method for the simultaneous detection of urinary AFM1, OTA, DON, DOM-1, FB1, α-ZOL and β-ZOL after multi-antibody immunoaffinity cleanup and reversed phase SPE. Similarly, a new multi-mycotoxin biomarker method based on pressurised liquid extraction (PLE) and HPLC-MS/MS has been developed for the detection of multiple mycotoxins[104]. This method even permits the measurement of mycotoxins in human and laboratory animal biological fluids and tissues, including urine, faeces, blood, breast milk, amniotic fluid, liver, kidney, and lung[104], and this will be very useful in assessing both short-term and long-term exposure. Similarly, an HPLC-MS/MS method with salting-out assisted liquid-liquid extraction has been developed for multi-mycotoxins and metabolites detection in both human and pig urine[105]. Recently, Rodriguez-Carrasco et al.[97], developed and validated GC–MS/MS method to determine levels of urinary mixtures of DOM-1, DON, 3-acetyldeoxynivalenol (3-ADON), fusarenon-X (FUS-X), diacetoxyscirpenol (DAS), nivalenol (NIV), neosolaniol (NEO), HT-2, T-2, ZEN, α-ZAL, β-ZAL, ZEN, α-ZOL, and β-ZOL. This method was applied in multi-biomarker detection of mycotoxins in human urine[97],[98]. In another recent study, LC-MS/MS with both 'dilute and shoot' approach after SPE and immunoaffinity clean-up was employed for multi-mycotoxin detection in urine samples[94]. DON-15-glucuronide, DON, ZEN, α-ZOL, β-ZOL, FB_1 and OTA biomarkers were concurrently determined. In Cameroun-West Africa, mixtures of biomarkers of 2 to 5 mycotoxins were detected in human urine[102],[106] whereas 52% of urine samples from a study in Italy had mixtures of biomarkers for DON, ZEN, FB_1 and OTA with 38% having co-occurrence of biomarkers for DON, ZEN and OTA[96]. An independent inter-laboratory evaluation of the performance of LC-MS/MS in multi-mycotoxin detection showed good and comparable concentrations for DON, DOM-1, AFM_1, ZEN and β-ZOL but showed unsatisfactory or questionable z scores obtained for FB1, OTA and α-ZOL[95].

Currently, most multi-mycotoxin detection methods are only carried out using urine that makes it difficult to ascertain long-term exposure and therefore, these methods should be optimised and validated for measurement in plasma/serum in order to check for long-term exposure. Similarly, efforts should be applied in synthesising more mycotoxin conjugates, including α-zearalenol glucuronide, β- zearalenol glucuronide, OTA glucuronide, and ochratoxin α glucuronide for future calibration and use in multi-mycotoxin methods[81] as these will give rise to novel ways of exposure assessment. In conclusion, the multi-biomarker methods are very useful as it has allowed the detection of analytes (e.g. DON-3-GlcA, DON-15GlcA and ZEN-14-GlcA) which were not previously detected using single biomarker methods and have improved the understanding of mycotoxins exposure assessment. In addition, it removes the burden encountered in extraction, clean-up and operating time needed in mycotoxins determination using single biomarker detection method as multiple mycotoxins can be analysed simultaneously in a single run using the same sample with increased sample throughput. However, one note of caution is that the sensitivity of these methods compared to some of the single biomarker methods already discussed is not always good, which may be an issue for some epidemiological studies of the effects of exposure.

4. Conclusions

Several mycotoxin exposure biomarkers are currently available, including AF-lysine adducts, urinary AFM1, AF-DNA adducts for AF, urinary FB1 for fumonisins, urinary DON and DON-glucuronides for DON, and potentially, urinary ZEN and α-ZOL for ZEN exposure. The improvements in analytical techniques for the simultaneous determination of multiple mycotoxins biomarkers in grains and biological samples promise assessment of multiple exposures to different mycotoxins in humans and animals and have also enabled the validation of different mycotoxin exposure biomarkers. However, efforts are still required to improve sample preparation procedures, inter-laboratory quality assurance and development of internal standards to ensure appropriate detection of biomarkers.

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Appendix 366

Appendix A. Summary of landmark studies on human exposure to mixture of mycotoxins using LC-MS/MS multi-biomarker methods. 367

References	Country	No. of subjects	Individuals examined	No of positive samples (%)	Analytes detected	Co-exposure in a single individual
Ahn et al. (2010)	Korea**	12	11 adults, 1 child	12 (100%)	AFM_1, OTA	AFM_1, OTA
Rubert et al. (2011)	Spain**	27	Adults	Not stated	AFG_2, OTA, DON	Not stated
Solfrizzo et al. (2011)	Italy**	10	Adults	10 (100%)	OTA, DON	DON, OTA
Warth et al. (2012a)	Austria**	27	Adults	26 (96%)	DON, DON-3-GlcA, DON-15-GlcA	DON, DON-3-GlcA, DON-15-GlcA
Warth et al. (2012b)	Cameroon**	175	145 HIV-positive adults, 30 HIV-negative adults	110 (63%)	AFM_1, OTA, FB_1, FB_2, DON, DON-3-GlcA, DON-15-GlcA, NIV, ZEN, ZEN-14-GlcA, α-ZOL	AFM_1, OTA, FB_1, FB_2, DON, DON-3-GlcA, DON-15-GlcA, NIV
Ediage et al. (2012)	Belgium**	40	Adults	9 (23%)	DON, OTA, OTα, 4-OH-OTA, ZEN, CIT, β-ZOL	DON, OTA, OTα, ZEN, β-ZOL
Shephard et al. (2013)	South Africa**	53	Adult women	53 (100%)	OTA, FB_1, DON, DON-3-GlcA, DON-15-GlcA, NIV, ZEN, ZEN-14-GlcA, α-ZOL, β-ZOL	OTA, FB_1, DON, DON-3-GlcA, DON-15-GlcA, ZEN, ZEN-14-GlcA, α-ZOL, β-ZOL
Rodriguez-Carrasco et al. (2014)	Valencia**	54	38 adults, 16 children	37 (68.5%)	HT-2, NIV, DON	DON-HT2, DON-NIV

Ezekiel et al. (2014)	Nigeria**	120	81 adults, 20 adolescents, 19 children	61 (50.8%)	AFM_1, DON, DON-15-GlcA, FB_1, FB_2, OTA, ZEN, ZEN-14-GlcA	Distribution of mycotoxins: 75% (46/61) had a single mycotoxin and 25% (15/61) had more than 1 mycotoxin [8 had 2 different mycotoxins, 5 had 3 different mycotoxins, 2 had 4 different mycotoxins, 7 had more than one mycotoxin/metabolite].
Cao et al. (2013)	China*	10	6 men, 1 pregnant woman, 3 lactating women	Not stated	AFB_1, AFB_2, HT-2, DON, DOM-1, ZEN, α-ZOL, β-ZOL, FB_1, FB_2, AFM_1, OTA, NEO, T-2 Triol,	3 samples had AFBs and FBs.
Abia et al. (2013)	Cameroon**		145 HIV-positive adults (29 male, 116 female), 30 HIV-negative adults	110 (63%)	DON, NIV, ZEN, OTA, FB_1, FB_2, DON-15-GlcA, DON-3-GlcA, ZEN-14-GlcA, α-ZOL, AFM_1	DON, OTA, NIV, FB_1, ZEN, AFM_1, FB_2
Ediage et al. (2013)	Cameroon**	220	220 children	160 (73%)	OTA, DON, AFM_1, FB_1, ZEN, α-ZOL, β-ZOL	Co-occurrence of 2, 3 and 4 mycotoxins was 35%, 5% and 5%, respectively.
Solfrizzo et al. (2014)	Italy**	52	26 males, 26 females	52 (100%)	DON, OTA, AFM1, FB_1, ZEN, α-ZOL, β-ZOL	Distribution of Mycotoxin mixtures in samples: 2 (DON, ZEN, FB_1, OTA, AFB_1); 27 (DON, ZEN, FB1, OTA); 20 (DON, ZEN, OTA); 1 (DON, ZEN, OTA, AFB_1); 2 (ZEN and OTA).
Gerding et al. (2014)	Germany**	101	Adult volunteers	87%	DON, ZEN, CIT, T-2, ENNB, DON-3-GlcA, ZEN-14-GlcA, DH-CIT	DON-ENNB-ZEN, DON-CIT-T-2, DON-CIT, DON-ZEN-DON-ENNB

Gerding et al. (2015)	Bangladish**, Germany**, Haiti**	287	95 adult Bangladeshis, 50 adult Germans, 142 adult Haitians	Bangladish (87%), Germany (80%), Haiti (68%)	DON, OTA, CIT, ENNB, AFM_1, FB1, α-ZOL, DON-3-GlcA, DH-CIT	DON-CIT-OTA-FB_1, DON-CIT-OTA, DON-OTA-ENNB, CIT-OTA-ENNB, CIT-OTA-FB1, AFM_1-CIT-OTA, AFM1-CIT-DON, ENNB-OTA, DON-OTA, CIT-OTA, CIT-FB_1, CIT-ENNB, AFM_1-CIT

*Urine, faeces, breast milk and amniotic fluid were analysed. **Only urine samples were analysed. **Abbreviations used**: NEO - Neosolaniol; AFB_1 - Aflatoxin B_1; DON - Deoxynivalenol; ZEN - Zearalenone; OTA - Ochratoxin A; NIV - Nivalenol; AFM_1 - Aflatoxin M_1; AFG_2 - Aflatoxin G_2; FB_1 - Fumonisin B_1; FB_2 - Fumonisin B_2; ZOL - Zearalenol; ZEN-14-GlcA - Zeralenone-14-O-glucuronide; α-ZOL-14-GlcA - α-Zearalenol-14-O-glucuronide; α-ZOL-7-GlcA - α-Zearalenol-7-O-glucuronide; β-ZOL-14-GlcA - β-Zearalenol-14-O-glucuronide; β-ZOL-16-GlcA - β-Zearalenol-16-O-glucuronide; DON-15-GlcA - Deoxynivalenol-15-glucuronide; DON-3-GlcA - Deoxynivalenol-3-glucuronide; DOM-1 - de-epoxy-deoxynivalenol; CIT - Citrinin; ENNB - Enniatin B; DH-CIT - Dihydrocitrinone.

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