Applied Research Note: Biomonitoring of mycotoxins in blood serum and feed to assess exposure of broiler chickens

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Primary Audience: Researchers, Broiler Producers, Nutritionists, Veterinarians

SUMMARY

Because European maximum guidance values of mycotoxins are only available for feed, mycotoxin exposure in animals is mainly monitored by feed analysis. However, proper sample collection is needed to ensure reliable results because of uneven distributions and disproportional spread of mycotoxins in feed which can hamper the evaluation of mycotoxin exposure in animals. A cross-sectional study was performed on 40 randomly selected broiler farms in Belgium. During a farm visit at the animal's age of 28 d, a pooled feed sample at the beginning and the end of the feed line was collected. Feed samples were analyzed by a validated multimycotoxin LC-MS/MS method. Moreover, serum samples were collected from 10 randomly selected chickens per farm. Serum concentrations of mycotoxins and major in vivo phase I metabolites were analyzed quantitatively, whereas the presence of phase II metabolites was determined in a qualitative approach by an UPLC-HRMS method. Deoxynivalenol (**DON**) was the most frequently occurring mycotoxin, being present in 74% of the feed samples, with an average concentration of 270 ± 171 μg/kg and a maximum concentration of 751 μg/kg in positive samples. Also the acetylated forms 3- and 15-acetyldeoxynivalenol (3 and 15ADON) were present in half of the samples, however, at lower concentrations (8 ± 3 μg 3ADON and 10 ± 7 μg 15ADON/kg). Only in 17.5% of the farms, DON was detected in serum samples at a mean serum concentration and standard deviation (SD) of 11 ± 19 ng/mL. The maximum serum concentration of 49 ng DON/mL was detected in broilers which were fed a diet that was contaminated with 191 μg DON/kg, whereas the maximum concentration of DON in feed was 751 μg/kg. Besides, 3 and 15ADON were only detected in 10% of the serum samples (max. 1.3 ng/mL). Sulfate conjugates of DON were only detected in a few serum samples. Qualitative screening for phase II metabolites of other mycotoxins showed similar results. Overall, correlations between feed and serum concentrations of all mycotoxins were lacking (R^2 = 0.18 for DON).

Key words: biomonitoring, mycotoxins, feed, serum, broiler chickens

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DESCRIPTION OF PROBLEM

Mycotoxins are secondary metabolites produced by different fungal species and are frequently found in food and feed. More than 400 different mycotoxins have been identified, with the most prevalent being aflatoxins (e.g., aflatoxin B1; **AFB1**), fumonisins (**FB**), zearalenone (**ZEN**), type B trichothecenes (e.g., deoxynivalenol; **DON**), type A trichothecenes (e.g., T-2 toxin; T-2), and ochratoxin A (**OTA**). These toxins are known to exert toxic effects in livestock, causing vague clinical symptoms such as disruption of growth and production parameters and suppression of the immunity (Bryden, 2012). Hence, maximum concentrations for AFB1, maximum guidance values for DON, ZEN, OTA, and FB and indicative values for T-2 in animal feed have been established in the European Union. Mycotoxin exposure can have a major negative impact on the poultry industry, as even low to moderate contamination levels affect organs in poultry such as the gastrointestinal tract and the liver as well as the immune system. This can result in an array of metabolic, physiological, and immunological disturbances and a reduced productivity of the birds (Bryden, 2012).

Being of major agroeconomic importance, assessment of mycotoxin exposure is crucial. Until now, mycotoxin exposure in animals is mainly monitored through feed analysis. However, feed analysis may disregard the so-called hotspots, which are local areas of higher contamination levels within the feed, and therefore complicate representative sample collection (Bryden, 2012). Hence, proper sampling is important to ensure more reliable results. An alternative to perform mycotoxin exposure assessment has been proposed, namely the direct measurement of biomarkers of exposure in biological matrices, such as blood, urine, or feces (Lauwers et al., 2019). Nevertheless, similar to feed analysis, biomonitoring through measurement of biomarkers in biological matrices comes along with certain disadvantages, for example, accomplishing an adequate time point of sample taking in relation to the oral ingestion of mycotoxins. Biomarkers for mycotoxin exposure in biological matrices can either be the mycotoxin itself or its phase I and II metabolites (Lauwers et al., 2019). Biotransformation into phase I and II metabolites is specific for each mycotoxin and toxicokinetic pathways of mycotoxins also differ between animal species (Broekaert et al., 2015). In pigs, for example, glucuronidation to DON-3-glucuronide is a major phase II metabolization pathway, while in broiler chickens sulfation to DON-3α-sulfate (**DON3S**) is the most important step during metabolization (Devreese et al., 2015).

Masked or modified mycotoxins in feed, as metabolites of fungi or plants, have been described and investigated as well, but legal regulations are mostly lacking. For example, the conjugated glucoside metabolite of DON (DON-3-glucoside) is fully hydrolyzed to DON in pigs, but no hydrolyzation to DON is seen in broiler chickens (Broekaert et al., 2017). Likewise, 3 and 15ADON are completely hydrolyzed to DON in pigs after oral absorption, whereas in broiler chickens, only 3ADON is completely hydrolyzed to DON but 15ADON is partially hydrolyzed. Hence, 15ADON is stated to be as toxic as or even more toxic than DON (Broekaert et al., 2015). Because modified mycotoxins can be toxic themselves and can be hydrolyzed either partially or completely in vivo to form their parent mycotoxin, increasing the exposure of these parent mycotoxins to the animal, novel biomonitoring methodologies should also take into account the modified mycotoxins.

Hitherto, studies on correlations between mycotoxin concentrations found in biological matrices compared with concentrations found in feed mostly investigated correlations for DON and/or ZEN in pigs, especially in urine. Only a few of these studies have been performed in broiler chickens. Therefore, the aim of this study was to comparatively evaluate mycotoxin exposure in broiler chickens under field conditions by feed analysis and by analysis of mycotoxins and their in vivo phase I and II metabolites in serum.

MATERIALS AND METHODS

Feed and Serum Sample Collection

Forty randomly selected Ross 308 broiler farms, located in Flanders (Belgium), were included in this study. Samples were collected

during a farm visit at day 28 of production. On each farm, 10 nonclinically diseased broilers were randomly selected from evenly distributed positions along the house without considering gender. The birds were euthanized by cervical dislocation by an experienced veterinarian. Immediately after euthanasia, blood was withdrawn by section of the jugular vein and collected into serum tubes, which were stored at $\leq -15°C$ until analysis. All chickens involved in this study were fed ad libitum. Blood sampling was executed at approximately the same time point (noon) on each farm on different days. In addition, a feed subsample of each 0.5 kg was collected on each farm at both the beginning and the end of the feed line and stored at $\leq -15°C$ until analysis. All farmers claimed that no mycotoxin binder was added to the feed; however, this could not be confirmed at the level of the feed mills. For each farm the subsamples were ground and subsequently pooled to one final sample prior analysis (1/1, m/m).

Serum Analysis

Serum samples were quantitatively analyzed for 24 mycotoxins and major *in vivo* phase I metabolites, namely DON, de-epoxy-deoxynivalenol 1 (**DOM1**), the sum of 3 and 15ADON, T-2, HT-2 toxin (**HT-2**), aflatoxin B1 (AFB1), aflatoxin M1 (**AFM1**), OTA, enniatin A, enniatin B, enniatin A1, enniatin B1, beauvericin (**BEA**), α-zearalenol (**AZEL**), α-zearalanol (**AZAL**), ZEN, β-zearalenol (**BZEL**), β-zearalanol (**BZAL**), tenuazonic acid, alternariol (**AOH**), alternariol mono-methyl-ether (**AME**), fumonisin B1 (**FB1**), and fumonisin B2 (**FB2**) using an in-house validated multimycotoxin liquid chromatography tandem mass spectrometry (LC-MS/MS) method, as described by Lauwers et al. (2019). A qualitative approach to detect phase II metabolites was performed using a validated ultraperformance liquid chromatography—high resolution mass spectrometry (UPLC-HRMS) method, as described by Lauwers et al. (2019) as well.

Feed Analysis

All 40 feed samples were quantitatively analyzed using a validated multimycotoxin LC-MS/MS as described by Monbaliu et al. (2010) for the simultaneous detection of 23 mycotoxins: AFB1, aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), aflatoxin G2 (AFG2), OTA, ZEN, FB1, FB2, fumonisin B3, T2, HT-2, nivalenol, 3ADON, 15ADON, diacetoxyscirpenol (**DAS**), fusarenon-X (**FusX**), neosolaniol (**NEO**), altenuene (**ALT**), AOH, AME, roquefortine-C (**ROQ-C**), sterigmatocystin, and enniatins (**ENN**) in feed.

Statistical Analysis

To reveal possible correlations between mycotoxin concentrations detected in feed and those detected in serum, a coefficient of determination (R^2) was calculated for each mycotoxin using Microsoft Excel.

RESULTS AND DISCUSSION

The mycotoxin concentrations detected in both feed and serum samples are shown in Table 1. No AFB1, HT-2, BEA, AZEL, AZAL, BZEL, BZAL, or AOH were detected in serum and no AFB1, AFB2, AFG1, AFG2, HT2, DAS, FusX, NEO, ALT, AOH, or ROQ-C were detected in feed samples. None of the mycotoxin concentrations detected in feed exceeded the European maximum (guidance) levels or indicative values. The most noticeable were the mean mycotoxin concentrations detected in serum being only a very small fraction of those detected in feed. Especially in chickens the oral bioavailability of most mycotoxins is low when compared with other animals. For example, DON was completely absorbed after oral administration to pigs, whereas only 10% (Broekaert et al., 2015) and 19.3% (Osselaere et al., 2013) of the mycotoxin was reported to be absorbed in the digestive tract of broiler chickens.

In this study, DON was the most frequently occurring mycotoxin, contaminating 75% of feed samples with a mean (±SD) and maximum mycotoxin concentration of 270 ± 171 μg/kg and 751 μg/kg feed, respectively. Similarly, in a global survey DON was prevalent in 37,940 of 59,107 animal feed samples collected between 2008 and 2017, which was 64% of positive samples and thus being the most frequently

Table 1. Observed mycotoxin concentrations in feed (n = 40 farms) and serum (n = 40, result per farm is the mean of 10 animals per farm) of broiler chickens.

	Feed				Serum			
Mycotoxin	% Positive samples	Mean ± SD in µg/kg	Maximum in µg/kg	Minimum in µg/kg	% Positive samples	Mean ± SD in ng/mL	Maximum in ng/mL	Minimum in ng/mL
NIV	5.0	32 ± 8	38	26	NA	NA	NA	NA
DON	75.0	270 ± 171	751	61	17.5	11.7 ± 19.1	48.8	1.6
3ADON	49.0	8 ± 39	14	5	10.0[1]	1.2 ± 0.1[1]	1.3[1]	1.2[1]
15ADON	51.0	10 ± 7	27	3	10.0[1]	1.2 ± 0.1[1]	1.3[1]	1.2[1]
FB1	40.0	147 ± 143	550	42	12.5	1.9 ± 1.1	3.7	1.1
FB2	20.0	83 ± 31	133	38	2.5	1.7[2]	1.7	1.7
FB3	10.0	56 ± 12	70	47	NA	NA	NA	NA
ZEN	ND	ND	ND	ND	5	3.1 ± 2.4	4.8	1.4
ENN	22.5	166 ± 221	640	14	ND	ND	ND	ND
T-2	5.0	11 ± 2	13	10	ND	ND	ND	ND
AME	2.5	31[2]	31	31	2.5	4.3[2]	4.3	4.3
ST	5.0	13 ± 1	13	13	ND	ND	ND	ND
OTA	ND	ND	ND	ND	5.0	2.0 ± 1.2	2.8	1.1
TEA	NA	NA	NA	NA	22.5	5.0 ± 2.9	10.1	1.8

Data are presented as percentages of positive samples, mean concentrations with standard deviations (SD) and maximum concentrations.

Abbreviations: 3ADON, 3-acetyldeoxynivaleol; 15ADON, 15-acetyldeoxynivaleol; AME, alternariol-monomethyl-ether; DON, deoxynivalenol; ENN, enniatins; FB1, fumonisin B1; FB2, fumonisin B2; FB3, fumonisin B3; NA, not analyzed; ND in feed, not detected or below decision limit (CCα); ND in serum, not detected or below limit of detection (LOD); NIV, nivalenol; OTA, ochratoxin A; ST, sterigmatocystine; T-2, T-2 toxin; TEA, tenuazonic acid; ZEN, zearalenone.

[1]Presented as sum of 3 and 15ADON.

[2]Only one positive sample.

occurring of all tested mycotoxins (Gruber-Dorninger et al., 2019). Remarkably, in this study, DON was only detected in serum samples of one of eight DON positive farms based on feed analysis. Accordingly, DON undergoes extensive biotransformation in broiler chicken and is eliminated rapidly (Devreese et al., 2015). The DON positive serum samples were characterized by an average serum concentration (±SD) of 11.7 ± 19.1 ng/mL. The highest serum concentration of 48.8 ng/mL was observed in chickens fed a diet containing 191 µg DON/kg feed, whereas the highest observed concentration of DON in feed in this study was 751 µg/kg feed, detected at another farm. Besides DON, the acetylated forms 3 and 15ADON were detected in feed samples of 50% of the farms, but at a lower mean (±SD) and maximum concentration of 8 ± 3 µg 3ADON and 10 ± 7 µg 15ADON/kg, and 14 µg 3ADON and 27 µg 15ADON/kg, respectively. By contrast, 3 and/or 15ADON were only detectable in 10% of the serum samples tested, at mean (±SD) and maximum concentrations of 1.2 ± 0.1 ng/mL and 1.3 ng/mL, respectively. Accordingly, after oral administration, a complete presystemic hydrolysis of 3ADON to DON and 75% hydrolysis of 15ADON to DON was observed in broiler chickens (Broekaert et al., 2015).

In contrast to the type B trichothecene DON, type A trichothecene T-2 was only present in the feed at 2 of 40 farms at mean and maximum concentrations of 11 ± 2 and 13 µg/kg feed. This 5% prevalence is in contrast to the results of the mycotoxin survey from Gruber-Dorninger et al. (2019), where 31% of the 21,036 feed samples taken in Central Europe were contaminated by T-2, although the mean concentration of 11 µg/kg was similar to this study. No T-2 has been detected in serum samples during screening. In accordance, after oral administration of T-2 to broiler chickens at a dose of 0.02 mg/kg BW no plasma levels of T-2 or HT-2 were detected (Osselaere et al., 2013) and a fast elimination was seen via excreta after administration of T-2 to broiler chickens.

During the metabolization of DON, de-epoxidation of the active epoxide group to DOM1 by the gut microbiota was observed as a

detoxification process in most animal species. No DOM1 was detected in broiler chickens after single bolus administration (Osselaere et al., 2013). In this study, DOM1 was detected in broiler chicken serum on 10% of the farms at mean (±SD) and maximum concentrations of 1.5 ± 0.2 ng/mL and 1.7 ng/mL, respectively. Sulfation has been discovered as a major metabolization pathway in chickens, with DON3S being the most dominant metabolic product of DON in chickens (Devreese et al., 2015). However, in this study, DON-15-sulfate (DON15S) was detected in only 1 of 400 serum samples, and no DON3S was observed. Although another biotransformation pathway of DON is glucuronidation, especially in mammals, only traces of DON-3α-glucuronide were detected after oral and intravenous administration of DON to broilers (Devreese et al., 2015). In accordance, no DON-glucuronide metabolites were detected in this study. Qualitative screening for phase II metabolites of other mycotoxins in this study revealed only very few positive samples, as well. During quantitative screening, phase I metabolite AFM1, a common metabolite of AFB1, was not detected in serum samples. Alpha-zearalanol was detected in serum samples of 4 of 40 farms at mean (±SD) and maximum concentrations of 2.9 ± 2.1 ng/mL and 5.9 ng/mL, respectively. Beta-zearalanol was present in serum samples of 2 of 40 farms at a mean (±SD) concentration of 8.3 ± 8.7 ng/mL and a maximum concentration of 14.5 ng/mL. No AZEL and BZEL were detected after chronic exposure to low doses of ZEN in this study. In accordance with literature, however, after single bolus of ZEN, AZEL and BZEL are the main metabolites of ZEN in most avian species (Osselaere et al., 2013).

Zearalenone was observed in serum samples of 5% of the farms at a maximum concentration of 4.8 ng/mL and a mean (±SD) concentration of 3.2 ± 2.4 ng/mL. Remarkably, no ZEN was detected in the matching feed samples. In addition, OTA was present in 5% of the serum samples but could not be detected in feed. Both could be associated with the sensitivity of the detection method, and differences in the limit of detection and decision limit in serum compared with feed (Monbaliu et al., 2010; Lauwers et al., 2019). Besides, OTA shows a long elimination half-life of almost 24 h, as well as an especially high plasma protein binding in broiler chickens, which prolongs the presence of OTA in the body. In addition, enterohepatic circulation has been suggested as a disposition process of OTA in broiler chickens (Devreese et al., 2018). These toxicokinetic parameters imply that broiler chickens tested in this study could have been exposed to OTA earlier in their lives.

In accordance with the high prevalence of the emerging *Fusarium* and *Alternaria* mycotoxins published in literature (Streit et al., 2013), ENN have been detected in 22.5% of the feed samples, while no traces have been detected in serum. In addition, AME could not be detected in serum samples but was present in feed samples of 1 of 40 farms. Tenuazonic acid could not be analyzed in feed but was observed in serum samples of 9 out of 40 farms and showed mean (±SD) and maximum concentrations of 5.0 ± 2.9 and 10.1 ng/mL, respectively. In a study of Streit et al. (2013), ENN, AME, and TEA contaminated 96, 82, and 6% of the 83 feed samples, respectively. The toxicity and toxicokinetic pathways of emerging mycotoxins are not yet fully investigated and no legislative regulation has been set up. Therefore, further research on in vivo toxicity and toxicokinetics of these emerging mycotoxins is needed in order to establish legal maximum or guidance levels.

Figure 1 shows a lack of correlation between concentrations of DON and its metabolites in feed and in serum samples in this study ($R^2 = 0.1768$). Correlations could not be calculated for other mycotoxins because no other mycotoxin was detected simultaneously in feed and serum on more than 2 farms. Exposure assessment studies often aim to find correlations between mycotoxins found in feed or biological matrices and impact on animal health. To the authors' knowledge, statistically significant correlations between clinical symptoms in broiler chickens and their exposure to mycotoxins, assessed by measurement of biomarkers of exposure, were not demonstrated yet. Nevertheless, some studies managed to demonstrate the involvement of mycotoxins by assessing biomarkers of exposure in certain diseases in pigs. In a study of Van Limbergen et al. (2017) e.g., a positive correlation (R = 0.52) between the concentration of DON in feed and in plasma between herds with and without tail necrosis in sows could be demonstrated. In contrast to biomarkers of

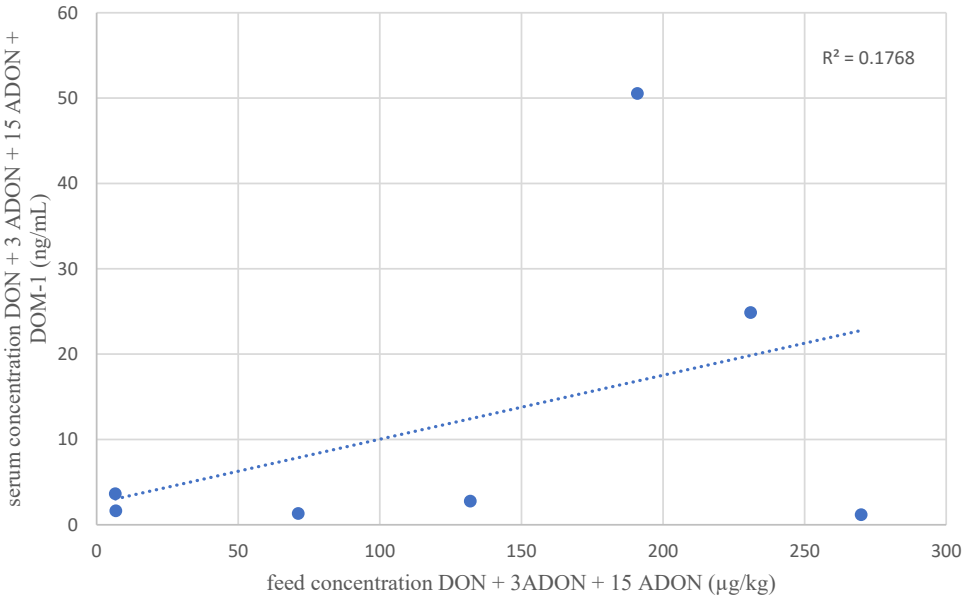

Figure 1. Serum concentrations of DON, 3ADON, 15ADON, and DOM1 in ng/mL in comparison with feed concentrations of DON, 3ADON, and 15ADON in µg/kg. R^2: coefficient of determination. Abbreviations: DON, deoxynivalenol; 3ADON, 3-acetyldeoxynivaleol; 15ADON, 15-acetyldeoxynivaleol; DOM1, de-epoxy-deoxynivalenol 1.

exposure, biomarkers of effect generally can give an estimation of the effect of mycotoxin exposure on animal health. Biomarkers of effect link biochemical, physiological, or behavioral alterations in the organism to possible adverse health effects. For mycotoxins, however, the only known biomarker of effect so far is the increase of the sphinganine/sphingosine ratio in blood, plasma, or tissue, which is seen after disruption of the sphingolipids biosynthesis caused by fumonisins (Bryden, 2012).

This study reveals how biomonitoring of mycotoxins based on determining their concentration and concentrations of related phase I and II metabolites in blood serum as biomarker of exposure can give an underestimation of the actual mycotoxin exposure in broiler chickens, probably due to the limited oral absorption and fast and efficient elimination of many mycotoxins in poultry species. However, even though the concentrations of mycotoxins detected in serum compared with those detected in feed showed a lack of correlation, serum analysis may show actual exposure to mycotoxins. Up to today mycotoxin exposure monitored by the means of feed analysis remains most suited. Further research on biomarkers of exposure, especially on biomarkers of effect, to assess mycotoxin exposure is needed to concur with the benefits of feed analysis on mycotoxins.

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DISCLOSURES

The authors declare no conflicts of interest.

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