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Federal Research Institute for Animal Health

34. MYCOTOXIN Workshop

Conference Abstracts

Braunschweig, Germany
14th May – 16th May, 2012

34^{th} Mycotoxin-Workshop

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The Organizing Committee would like to thank all staffs of the Institute of Animal Nutrition, Friedrich-Loeffler-Institute, Federal Research Institute of Animal Health, Braunschweig, especially **Ulrike Brezina**, **Mohammad Ebrahem**, **Inga Rempe** and **Janine Winkler**. Without their help, this workshop would not have been possible!

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Development of a risk-based sampling concept for official mycotoxin laboratories

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At the Chemical and Veterinary Laboratory Stuttgart, known as CVUA Stuttgart (one of 4 official food control and animal health laboratories in Baden Wuerttemberg (BW)), about 26,000 samples are analysed each year. To assure the best possible consumer protection, the effectiveness of selecting appropriate samples to analyze is becoming more important, given the decrease in human and financial capital in the administration of BW. Therefore, a risk-based state-wide sampling coordination process has been established in BW (based on Roth, Renz, 2005).

Mycotoxin sampling has always been unpopular, due to the time-consuming sampling procedure required by the Commission regulation (EG) No. 401/2006 and, among other things, low rates of exceeding MRLs compared to other analytical fields. This has led to occasional calls for a decrease in the quantities of mycotoxin samples. To promote acceptance and prevent further discussions about the necessity and suitability of mycotoxin samples, the official experts at CVUA Stuttgart have developed an efficient, risk-based mycotoxin sampling concept.

For the concept criteria of mycotoxins in foodstuffs like e.g. toxicity, objective relation of the risk of mycotoxins compared to other food contaminants, occurrence, relevance in foodstuffs, reports of exceeded MRLs, results from border controls and validation of self-monitoring of the companies have been taken into consideration.
With this in mind, the pragmatic risk-based sampling concept for mycotoxin laboratories has been developed, which poses an approximation of a first step. The simple formula is: one sample of a mycotoxin risk product per known company per year and 2 samples of a mycotoxin high risk product, like pistachios, per known company per year. In addition, samples originating from monitoring projects or random market controls are to be collected. This leads to a sample quantity of 2,000 mycotoxin samples in BW per year.

The criteria and their priorities for the risk-based sampling concept and difficulties in mycotoxin sampling will be presented and discussed, along with the question of consumer protection vs. ease of sampling.
The question is, do you advance consumer protection by taking e.g. many GMO samples, with unknown risks, due to an easy sampling procedure and a current high interest of the media? Or do you act at the cost of consumer protection by neglecting mycotoxin samples, due to the great effort of sampling even when the risks are known?

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Is quality management in ISO 17025 accredited mycotoxin laboratories a challenge?

Simone Staiger, Scarlett Biselli

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At first sight a lot of standard methods are available to perform mycotoxins. Particularly for aflatoxins and ochratoxin A the standard procedures with immunoaffinity column clean-up and HPLC-FL detection are well known and accepted in the whole world. It seems to be easy to take one of these standard operation procedures and transfer it to a 17025 accredited laboratory. For ISO, EN or DIN methods it is received not to carry out a full validation. Nevertheless the accredited lab has to show, if its performance for repeatability and reproducibility is comparable or better than reported in the norm.
Besides the validated matrices in the standard methods most laboratories have to extend their scope of matrix and this is often connected with deviations to the standard protocol which have to be validated. The detection of aflatoxins is affected by the highest number of different commodities. A laboratory which is not focused on a special class of goods will be faced with hundreds of different commodities. ISO 17025 defines the validation of analytical procedures in some aspects not precise and laboratories have to specify their criteria and protocols with regard to EC 401/2006. Quality parameters like recovery rate, coefficient of variation and expanded uncertainty of measurement depend on the matrix. This correlation causes high efforts in validation work. Especially for complex foods a practicable procedure might be the definition of matrix groups depending on their components. For instance bakery products could be divided into classes with high and low sugar contents or a categorisation according their cereal species could be taken into account. But validation work is not the only issue in accredited laboratories. On the one hand claims of the accreditation bodies have to be met and on the other hand requirements of customers. Major laboratories are expected to have well structured quality concepts.

Development and application of a rapid multi-biomarker LC-MS/MS method for human mycotoxin exposure assessment

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Mycotoxins regularly occur in grains and processed foods worldwide. Therefore, it is crucial to have accurate and sensitive tools for the assessment of human exposure to these natural contaminants. As individuals can be exposed to a variety of mycotoxins at the same time, there is a demand for estimating exposure by analysis of suitable biomarkers simultaneously. This paper reports on the development of a simple and time effective LC-MS/MS method for the quantitative measurement of nine major food mycotoxins including six key metabolites in human urine. Deoxynivalenol (DON), deoxynivalenol-3-*O*-glucuronide (D3GlcA), deoxynivalenol-15-*O*-glucuronide (D15GlcA), de-epoxy deoxynivalenol (DOM-1), nivalenol (NIV), T-2 toxin (T-2), HT-2 toxin (HT-2), zearalenone (ZEN), zearalenone-14-*O*-glucuronide (Z14GlcA), α- and β-zearalenol (α- and β-ZEL), fumonisins B_1 and B_2 (FB_1, FB_2), ochratoxin A (OTA) and aflatoxin M_1 (AFM_1) were determined in a single 18 minute LC-MS/MS run without the need for any clean up. The validation was carried out at five concentration levels in the range of 0.005-40 $\mu g\ l^{-1}$ depending on the analyte's detector response and expected urinary concentration levels. Recoveries between 78 and 119% and 1-13% RSD and 2-17% RSD intra- and interday precision, respectively, were satisfactory for all analytes.

The suitability of the chosen biomarker approach and the applicability of the method were demonstrated by the analysis of urine samples obtained from individuals from Austria and Cameroon. In one sample from Cameroon six analytes (AFM_1, DON, D15GlcA, NIV, FB_1, and OTA) were simultaneously detected, emphasizing the great importance of the developed method for overall mycotoxin exposure assessment. In the 27 human urine samples from Austria DON and its glucuronide conjugate(s) were detected. Free DON was found in six of these samples while conjugated DON-glucuronide(s) were detected in 26 samples. In contrast to previous findings we were able to determine and chromatographically separate two closely eluting DON-glucuronides in naturally contaminated urine samples. A smaller peak was identified as D3GlcA by using a synthetically produced standard to whereas a second, larger peak was tentatively identified as originating from D15GlcA. These new findings clearly demonstrate the great potential of the newly developed biomarker method to critically assess exposure of humans and animals to multiple naturally occurring mycotoxins.

Analysis of citrinin in human biological fluids: blood and urine

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Citrinin (CIT) is a mycotoxin mainly produced by species of the genera *Penicillium* and *Monascus*. The kidney is the major target organ of CIT toxicity [1, 2], and both additive or synergistic effects have been described in combination with ochratoxin A (OTA). CIT occurs in a wide spectrum of food commodities and is frequently found together with OTA [3, 4]. This indicates that humans can be exposed to CIT through food intake. Animal data showed that CIT is detectable in serum and actively excreted in urine [5]. In humans CIT-biomonitoring studies have not yet been performed. Therefore, the aim of this study was to develop suitable analytical methods for the analysis of CIT in blood plasma and urine and to assess exposure of humans to this mycotoxin.
Urine samples were cleaned up using two analytical methods: C18 solid phase extraction (SPE) and immunoaffinity extraction (CitriTest®). Analysis of the extracts was performed by high performance liquid chromatography (HPLC) and fluorescence or tandem mass spectrometry (MS) with limits of quantification (LOQ) of 50 ng/L for the MS technique. Both methods were fully validated and compared in terms of efficiency. Finally, the methods were applied to a small human cohort.
Blood plasma was diluted with acetonitrile. The denatured proteins were separated by centrifugation and the supernatant was evaporated to dryness. After reconstitution with methanol the sample was filtered and injected into the HPLC. Detection was performed by tandem MS. The LOQ is about 100 ng/L.
The validation of the urine methods show excellent recovery rates for both clean-up methods. However, immunoaffinity extraction offers a higher clean up efficiency over SPE-C18 columns. By comparing the detection systems, lower limits of detections can be reached with tandem MS than with fluorescence. The application of this method to a small cohort revealed that CIT is present at very low concentrations (LOQ) in urine. In blood plasma, CIT was detected in the majority of the samples. Interestingly, these samples were tested also positive for OTA.
This is the first study in which the occurrence of CIT in biological human samples is reported. Moreover, preliminary data confirmed the co-occurrence of two nephrotoxic mycotoxins in human plasma: CIT and OTA.

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Improved positive electrospray ionisation of patulin by adduct formation: Usefulness in multi-mycotoxin LC-MS/MS analysis

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Patulin is a mycotoxin produced by several fungal species, mainly by *Penicillium* spp. and *Aspergillus* spp. Since patulin-producing fungi are widely spread, this toxin has been detected in food, feed and in mouldy water-damaged dwellings. Co-occurrence of patulin with other mycotoxins has also been reported (Nielsen, 2003). Patulin is commonly analysed by liquid chromatography with UV detection. Liquid chromatography coupled to mass spectrometry (LC-MS/MS) is considered as a more specific tool for mycotoxin detection and confirmation. However, the implementation of this technique for the determination of patulin, especially in the context of multi-mycotoxin analysis, is limited due to ionisation problems.
In this study, the effect of different solvents, mobile phase additives and pH on the ionisation and fragmentation pattern of patulin was investigated. The preliminary results showed that under alkaline conditions and using methanol as organic modifier, an intense and stable signal for the methanol-adduct of patulin was obtained in the positive electrospray ionisation mode (ESI^+). The fragmentation of this protonated methanol-adduct gave a strong and stable product ion signal. The product ion spectra were overall more useful than those obtained with the protonated or the deprotonated molecule. These findings indicate the possibility of using the protonated methanol-adduct of patulin for its identification and quantification by LC-MS/MS.
This presentation will highlight the generation of a stable and useful patulin signal by adduct formation as well as the optimisation of this signal by means of experimental design. Furthermore, the successful inclusion of this toxin in a multi-mycotoxin LC-ESI^+-MS/MS method and the application of this method to different analytical challenges will be demonstrated.

Reference

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Moniliformin – first application of an isotopically labeled standard for HPLC-FTMS analysis

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Moniliformin, a mycotoxin produced by different *Fusarium* species, occurs as worldwide contaminant of different cereals [1]. So far, no legal limit for this toxin is published, so that the aim of the current work was to develop a sensitive and robust analytical method to improve the feasibility of monitoring the human exposition to moniliformin by contaminated food.
The chosen approach for the development of a quantitation method for moniliformin in low concentration ranges was based on the use of high performance liquid chromatography coupled to high resolution mass spectrometry (HPLC-FTMS) as by FTMS a high sensitivity and selectivity is achieved.
To compensate matrix effects during ionization, $^{13}C_2$-moniliformin (Figure 1), which was recently synthesized in our group in a four step synthesis using trichloroacetyl chloride-1,2-$^{13}C_2$ as ^{13}C-source [2], was added just before analysis. The developed method uses a sample preparation that was optimized according to literature data [3] and has a recovery rate of 75.3%, a limit of detection (LOD) of 0.7 µg/kg and a limit of quantitation (LOQ) of 2.5 µg/kg (determined in corn grits). With this method 23 cereal-based samples were analysed for their moniliformin content resulting in levels from not detectable up to 126 ± 12.2 µg/kg [4].

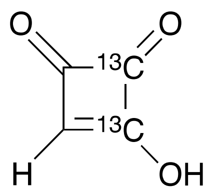

Figure 1: Structure of the isotopically labeled $^{13}C_2$-moniliformin

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Sample preparation for the quantification of beauvericin and enniatins in vegetables and cereals

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Introduction: Beauvericin and enniatins are cyclic hexadepsipeptides produced by a wide range of *Fusarium* species which colonize various cereals and vegetables. These mycotoxins have a nonpolar character and act as cation chelating agents, ionophores and antibiotics. In this study we optimized the sample extraction, defatting and MS analysis of beauvericin and enniatins.

Material and methods: To determine recovery rates, maize, rice, wheat, potato, asparagus and tomato were spiked with 1 µg/g of beauvericin, enniatin A, enniatin A1, enniatin B, and enniatin B1 and extracted with different solvents. Matrix effects were studied by spiking extracts of clean matrix with defined amounts of mycotoxins. Different organic solvents for defatting of the extracts were compared. The efficiency of defatting was evaluated with the help of lipophilic dye Sudan III. Mycotoxin content was determined by an optimized LC-MS/MS method on a 500-MS ion trap (Varian, Darmstadt, Germany).

Results and Discussion: The data show that depsipeptides can be extracted from vegetables and cereals with a high recovery rate with all tested solvents, whereas the influence of matrix on ionization varied strong among the different plants. Recovery rates and matrix effects were determined. Defatting extracts for beauvericin and enniatins analysis is known to be difficult because of loss of toxins to the hexane phase. Modification of hexane phase as extraction solvent was suggested as a means to avoid this problem. We found, however, that the efficiency of defatting was strongly reduced. We therefore optimized the composition of water/methanol phase as well as hexane/dichloromethane extraction solvent for high defatting efficiency and minimized analyte loss.

Molecular methods in Food mycology: Development and Implementation of a mycotoxin microarray

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Food and feed are prone to contamination with filamentous fungi and their mycotoxins. FAO estimates that up to 25% of cereal based food stuff is contaminated and has to be rejected. This fact shows impressive the importance of mycological research for economic and health reasons. Chronic ingestion of some of the mycotoxins, which all belong to the group of secondary metabolites, could lead to several health defects like allergies or even cancer.

In previous experiments could be shown, that there is a strong relationship between mycotoxin specific gene expression and active toxin biosynthesis. For this reason we developed a DNA based Microarray, which covers the complete mycotoxin biosynthesis genes of the aflatoxin, fumonisin, ochratoxin, patulin and the trichothecene type A and B gene cluster.

With this tool it is e.g. possible to identify mycotoxin producers using a strain specific gene expression pattern, and to monitor single genes whose expression is crucial for a specific chemotype, like the *tri*3 in case of 15-acetylated DON in *F. graminearum*. Another approach is the possibility to analyze gene expression in mycotoxigenic fungi during storage or infection of a host plant. Together with translational data (protein level) and secondary metabolomic data, the knowledge on transcriptional level could lead to a comprehensive understanding of the molecular background of growth and mycotoxin biosynthesis of different fungi which could lead to the development of prevention strategies for safer food and feed.

Immunochemical approach for masked mycotoxin determination

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Together with mycotoxins, which are secondary fungal toxic metabolites, many of their conjugated forms, so-called 'masked mycotoxins', generated by plant metabolism or by food processing, were found. During these processes mycotoxins (for example, zearalenone (ZEN)) are converted into more polar forms via conjugation with sugars, amino acids or sulphate groups. These mycotoxin derivatives are characterized by different chemical properties, but the most threatening is that these compounds possibly can be hydrolyzed to their precursors in the digestive tracts of human and animals and cause unexpected high toxicity of food and feed.

So far, two approaches (direct and indirect) are being employed for the determination of masked mycotoxins. The direct approach makes use of chromatographic separation followed by fluorescence, ultraviolet or mass spectrometric detection of the intact masked forms. The indirect approach is based on the conversion of the masked forms to their parent mycotoxins. The total amount of these parent forms is determined before and after conversion. Conversion of the masked forms to the parent analyte can be performed through acidic, basic or enzymatic hydrolysis. The amount of masked forms is calculated by subtracting the measured amount of parent analyte before conversion from the total measured amount of parent analyte after conversion.

In this study, enzymatic and acidic techniques of zearalenone-4-glucoside (Z4G) hydrolysis were evaluated. For acidic cleavage, trifluoromethanesulfonic acid (TFMSA) was used. The main advantage of TFMSA-mediated deglycosylation is the removal of carbohydrate chains regardless of the type of linkage and composition. Different concentrations of TFMSA under different time-temperature conditions of deglycosylation were investigated. Percentage of acidic hydrolysis was around 70%. For enzymatic cleavage, 7 different kinds of glycoside hydrolases were compared. Optimization involved choice of optimal amount of each enzyme and time-temperature-medium conditions. The best result was obtained for glucosidase from *Aspergillus niger*: it allowed to hydrolyze more than 80% of Z4G in real samples. Optimal reaction time was 30 min.

Immunochemical methods allow to determine mycotoxins only by the indirect approach, because currently, no antibodies specifically developed against conjugated mycotoxins are available. Two immunoassay formats for the detection of ZEN in cereal samples were employed: enzyme-linked immunosorbent assay (ELISA) and gel-based column test-method. The developed ELISA technique was characterized with good sensitivity (IC 50 value was 0.4 ng mL^{-1}) and repeatability. The cut-off level for the qualitative column test-method was based on the maximum level for ZEN in unprocessed cereals (100 microgram kg^{-1}) established by the European Commission.

As confirmatory method, liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) was used. Good correlation was found between the LC-MS/MS results and the results obtained with the different immunoassay methods.

The use of flow-cytometry and planar array based formats for multiplexed immuno-detection of mycotoxins in cereals and beer

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In our research we used the xMAP technology developed by Luminex to develop a multiplex suspension array immunoassay for simultaneous detection of the mycotoxins Aflatoxin B_1 (AFB_1), Fumonisin B_1 (FB_1), Deoxynivalenol (DON), Ochratoxin A (OTA), Zearalenone (ZEA) and T-2 toxin (T-2).
The paramagnetic beads (6.5 µm) used in the presented research are internally dyed with two or three fluorochromes to create up to 500 unique bead sets. The beads are excited with red light for classification. This creates the possibility for using just one reporter molecule, which is the fluorescent protein R-Phycoerythrin (R-PE), excited by green light. The bead sets are carboxylated which facilitates the easy covalent coupling of antibodies to the bead surface. Detection molecules were created by the conjugation of mycotoxins to R-PE. In this way we created unique reporter molecules for each assayed mycotoxin.
Both xMAP suspension and planar array formats were used during the development of this fast direct inhibition immunoassay. The FlexMap 3D flow cytometer is a bead-dedicated flow cytometer capable of characterising 500 unique analytes in a single well. The MagPix system is a planar array platform which is also based on the xMAP technology. The machine uses a monolayer of paramagnetic beads captured by a magnetic source in a flow chamber and LED lights combined with CCD imaging and therefore technically differs from the flow cytometry formats. It can detect 50 analytes in a single well.
Initially, this assay was a 3-plex for the detection of FB_1, ZEA and OTA. Using this 3-plex, wheat and maize reference materials were analysed. The accuracy for OTA and ZEA was satisfactory, but for FB_1 high overestimations were observed (10-fold). In a later stage AFB_1, DON and T-2 were added and a large collection of international beers from various sources was screened. Preliminary results show high concentrations of DON (up to 700 ppb), but also for Aflatoxin B_1 (up to 3 ppb) in certain beers. The confirmation of these results by LC-MS/MS is still pending. We also checked for cross-interactions of the used antibodies with known metabolites and some masked mycotoxins.
In the future this assay can be easily extended with other mycotoxins of interest, provided that relevant antibodies are available.

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Early prediction of mycotoxin contamination in maize based on detection of volatile organic compounds

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Volatile organic compounds (VOCs) possess high vapor pressure and evaporate easily at room temperature. These carbon-based compounds released by living organisms belong to various chemical classes like alcohols, esters and terpenoids. Due to their volatility, VOCs can travel large distances in heterogeneous environments. They are known to mediate various biotic interactions. VOCs of a certain plant-pathogen-system can be divided into pathogen-derived VOCs, such as trichodiene as a precursor of trichothecenes produced by *Fusarium* spp., and plant-derived VOCs. *De novo* synthesis of plant-derived VOCs occurs upon abiotic and biotic stress, such as high temperature, drought stress and attack by pathogenic fungi and insects.

To investigate the changes in the profile of emitted VOCs between healthy and fungal-infested maize as well as the production of mycotoxins, we cultivated commercial hybrid maize (KWS, Ronaldinio) under greenhouse conditions. Maize cobs were infected at flowering stage (BBCH 65) with *Fusarium graminearum* (both deoxynivalenol (DON) and nivalenol (NIV) chemotypes), *F. verticillioides* and *F. subglutinans* as well as mixtures of *F. graminearum* and *F. verticillioides* by injection of spore suspensions into the silk channel. Sample collection was carried out at 24 dpi. For inoculations with *F. graminearum* (DON chemotype) and *F. verticillioides*, time series were carried out from 4 dpi to 24 dpi at 6 time points. Furthermore, we investigated the production of VOCs under *in vitro* conditions, by cultivation of *F. graminearum* and *F. verticillioides* strains on autoclaved maize and rice kernels for 7 days.

A destructive static headspace sampling (solid-phase microextraction (SPME)) as well as a non-destructive dynamic headspace sampling (open-loop stripping method using activated charcoal cartridges as volatile traps) were carried out to collect volatiles from maize cobs. *In vitro* cultures were sampled by SPME technique only. Collected volatile samples were analyzed by gas chromatography coupled with mass spectrometer; flame ionization detector (FID) was used for quantification. Identification of VOCs was carried out by mass spectra comparison with NIST 2008 as well as Adams and Wiley mass spectral libraries. A multiple comparison test after Kruskal-Wallis was carried out to determine statistical differences between treatments. The level of mycotoxin contamination was analysed with HPLC-MS. Fungal biomass was determined by quantitative real-time PCR.

We observed considerable differences in the composition of volatile organic compounds between healthy and infected plants. A various number of VOCs, such as green leaf volatiles, known fungal volatiles as well as a large number of plant sesquiterpenes (alpha- and beta-selinene, beta-macrocarpene etc.) were detected *in vivo* within 4 to 8 dpi. The production of these VOCs continued until early milk ripeness (24 dpi). These VOC profiles were not found *in vitro*. The mycotoxin DON was detected at an early infection stage of 4 dpi while the production of zearalenone and fumonisins were observed at later time points. Volatile biomarkers of mycotoxin contamination appear particularly useful for early detection of fungal infection before disease symptoms become visible.

Functional characterization of *Brachypodium distachyon* UDP-glucosyltransferases and candidate deoxynivalenol detoxification genes from crop plants

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Production of deoxynivalenol (DON) is a virulence factor of *Fusarium graminearum*. In turn, resistance to *Fusarium* spreading is largely determined by the ability to convert DON into the non-toxic conjugate DON-3-O-glucoside. The identification of the first UDP-glucosyltransferase (UGT) gene from a monocotyledonous plant encoding an enzyme with this activity (Schweiger et al. 2010) is a starting point to identify the relevant genes in other crop plants. Since the wheat and barley genomes are not yet sequenced, we have investigated conservation of the HvUGT13248 gene in the sequenced genome of the monocot model species *Brachypodium distachyon* (Bd), which is closely related to wheat, and in the genomes of rice and Sorghum. The gene family of Bd UDP-glucosyltransferases (UGTs) consists of 177 predicted genes. We characterized the cluster of 6 Bd genes which have the highest sequence similarity with HvUGT13248. Only two out of the six candidate genes conferred DON resistance when expressed in yeast. We also tested members of the corresponding rice cluster and the single copy Sorghum gene. While some gene products were inactive, others showed quantitative differences in the capability to detoxify DON and also nivalenol (NIV). Since it is unknown which NIV-glucoside is formed, synthesis of reference substances was initiated. Overexpression of HvUGT13248 in *Arabidopsis thaliana* conferred DON resistance, and led to intracellular accumulation of D3G. In summary, clustered UGT genes seem to evolve rapidly, so that it is nontrivial to identify true orthologs in crop plants. Sequence similarity is not sufficient to predict substrate specificity. Overexpression of a DON detoxifying UGT gene is a valid strategy to increase DON resistance in plants.

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Occurrence of deoxynivalenol and deoxynivalenol-3-glucoside in durum wheat

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Fusarium graminearum and *F. culmorum* are the main causal agents of Fusarium Head Blight (FHB), one of the most common disease of small grain cereals[1]. FHB has been permanently present in Italy since 1995, especially in the north-central regions, with variable outbreak severity related to the year, area and cultivars. The infection may lead to the accumulation of mycotoxins in grains, in particular deoxynivalenol (DON) and its 3- and 15-acetyl-derivatives. DON is a trichothecene mycotoxin that inhibits DNA, RNA and protein synthesis. It is responsible for hemorrhagic and anorexic syndromes as well as neurotoxic and immunotoxic effects in mammals[2].
The production of DON by Fusarium spp. is believed to play a significant role in pathogenesis: resistant cereal lines inoculated with pathogens of the genus Fusarium contain lower amounts of DON in the grain in comparison with susceptible ones[3]. Recently, it has been shown that cereal crops exposed to DON infection are capable of detoxifying this mycotoxin. In this context, one major pathway is the conjugation of DON to a glucose moiety giving rise to deoxynivalenol-3-glucoside (D3G)[4]. Although very few is known about the toxicological relevance of D3G in comparison to its parent compound, it has been suggested that its production plays a key role in the FHB resistance mechanism in wheat and barley.
To our knowledge, no data have been provided so far about D3G occurrence in durum wheat (*Triticum durum* Desf.), which is a key cereal for the Italian food production industry. Thus, the aim of this work is the evaluation of the DON and D3G occurrence in durum wheat samples (n = 150) collected in 2010 from 3 areas located in Northern-Central Italy. The extraction procedure was optimized for DON in durum wheat by chemometry-assisted Design of Experiment (DoE) followed by UPLC-ESI-MS analysis. Validation parameters were given for the main target analytes. Results were statistically compared and analysed in order to verify potential correlations with the pathology.

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Determination of deoxynivalenol, deoxynivalenol-3-glucoside and 3-acetyl-deoxynivalenol in 374 beer samples

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After water and tea, beer is the third most popular beverage worldwide. Since beer is brewed from malted cereal grains, it is known to be potentially contaminated with mycotoxins. Some studies have shown that along with the *Fusarium* mycotoxins deoxynivalenol (DON) and 3-acetyl-DON (3ADON) also the conjugated mycotoxin deoxynivalenol-3-glucoside (D3G) can be found in beer.

The aim of this work was to develop the first triple quadrupole LC-MS/MS based method for the determination of DON, D3G and 3ADON in beer and to perform an in-house validation. Sample preparation included degassing, precipitation, evaporation and reconstitution. Since different kinds of beer exist and method performance parameters will likely differ, the samples were classified into pale, wheat, dark, bock and non-alcoholic beers, as well as shandies, and all six matrices were validated. To obtain method performance parameters, three individual beers for each category were spiked at eight levels prior to sample preparation.

The relative standard deviation of the apparent recovery was excellent (4 to 16%) for all analytes and types of beer. Limits of detection were in the sub- or low-ppb range. Apparent recoveries of 39-69% for D3G, 60-90% for DON and 96-124% for 3ADON were obtained for the different types of beer, with dark and bock beers being the most difficult matrices.

This validated method was applied to the analysis of 374 beer samples from 38 countries. In total, 348 and 289 out of the 374 beer samples contained D3G and DON concentrations above the limit of detection. The determined average concentrations of all beer samples regardless the category were 6.9 µg/L for D3G and 8.4 µg/L for DON. Not surprisingly, bock beers with a higher gravity also contain more mycotoxins on average. The highest contamination was observed in a single pale beer from the Austrian market with 81 µg/L of D3G and 89 µg/L of DON, underlining the importance of this study.

Screening of mycotoxins in tomatoes, onions, bell peppers, soft red fruits and derived tomato products with LC-TOF-MS

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Risk assessment studies and legislation are available for multiple mycotoxins such as aflatoxins, ochratoxin A, deoxynivalenol, zeoralenone, fumonisin, T-2 and HT-2 toxin in cereals, maize and dried plant products (e.g. nuts, spices and dried fruits) *(1)*. Also, patulin in apple and apple products is widely documented *(2)*. However, limited data are available for mycotoxins associated with fresh produce and their derived products. Recently, EFSA published a report on the status of fresh produce and possible mycotoxins *(3)*. It indicates the need for further research on hazard identification and risk assessment studies. A multi screening method was developed with LC-TOF-MS to screen moulded fresh produce (tomatoes, onions, sweet bell peppers and soft red fruits) from different countries (Belgium, Spain, India, Brazil, South Africa, Egypt) for the presence of a multitude of mycotoxins possibly associated with fresh produce (alternariol (AOH), alternariol monomethyl ether (AME), ochratoxin A (OTA), fumonisins (FB1, FB2, FB3)). In total 320 moulded products were investigated. AOH and AME was found in all four types of fresh produce (respectively 30 and 25% prevalence). Fumonisins and ochratoxin A seems not to play a major role as a mycotoxin hazard, with exception of FB2 in moulded bell peppers from India. In total 173 samples of derived tomato products (such as ketchups, concentrates, dried tomatoes) were screened. AOH and AME were respectively found in seven and five derived tomato products (concentrates and purees). The presence of these *Alternaria* mycotoxins in both fresh produce and derived food products urgents the need for a dietary exposure risk assessment, with quantitative mycotoxin concentration data and consumption data.

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Fusarium species and mycotoxins in silage maize and the effect of cropping factors

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Maize is frequently infected by *Fusarium* species producing mycotoxins. Numerous investigations have focused on grain maize, but little is known about *Fusarium* species in the entire plant used for silage. Furthermore, mycotoxins persist during the ensiling process and thus endanger feed safety. In the EU and Switzerland, guidance values exist for feed, however, silage is not controlled since it is fed directly on the producing farm. In the current study, we analysed silage maize samples for the incidence of *Fusarium* species and mycotoxins. A total of 40 samples per year was collected throughout two years from growers' fields in the Swiss cantons Berne and Aargau. In Aargau, a pre-harvest visual disease rating showed few disease symptoms. Nevertheless, detection using species specific morphological criteria on various plant parts revealed a *Fusarium* infection of every sample with an average incidence of 64% and fifteen different *Fusarium* species in 2011. Similarly, the average infection rate was 58% and fourteen different species were detected in Berne. Both the year and the geographical origin had an effect on the dominating species. No infection preferences for certain plant parts were observed in 2010, but the colonization between kernels and stalks differed significantly in Berne in 2011. Mycotoxin concentrations measured by both LC-MS/MS and ELISA often exceeded guidance values for complete and supplementary feeding stuffs for different animal species and age. For example 42% of the samples exceeded the deoxynivalenol (DON) guidance value for calves and 84% were inappropriate for feeding pigs in Berne in 2010. Every fourth sample exceeded the zearalenone (ZEA) guidance value for dairy cattle. Samples from Aargau were analysed for further toxins. Zearalenone, nivalenol, HT-2 and T-2 toxin, acetylated DON and fumonisins were detected in descending order of concentration. The high diversity of the *Fusarium* species observed and various agricultural and weather conditions represent a challenge to identify cropping factors that influence disease incidence. Results from a generalized linear regression model containing three cropping factors to explain DON contamination will be presented and general recommendations to avoid mycotoxin contamination in silage maize will be discussed.

Tab.1: Samples exceeding guidance values (%)

Toxin	Guidance value	Consumer	Aargau n=19	Berne n=21
DON	900 ppb	pigs	68	84
	2000 ppb	calves < 4 month	21	42
ZEA	250 ppb	sows, fattening pigs	16	32
	500 ppb	calves/dairy cattle	0	26

Fusarium mycotoxins in silage maize samples from two different cantons in 2010: percentage of samples exceeding guidance values after EU regulation 2006/576 for the two major mycotoxins

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Pentahydroxyscirpene – detection, isolation and structure elucidation of a new mycotoxin

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Although a large number of trichothecene type mycotoxins are described in the literature and are structurally characterized, there are still numerous possibilities for novel compounds. This is mainly because of their backbone structure which provides an ideal scaffold for different functional groups and therefore leads to many theoretically possible toxins.

During a routine purity check of a nivalenol (NIV) sample by NMR, a substantial amount of a structurally closely related impurity was identified. Since the polarity was slightly different from that of NIV the substances were purified via normal phase liquid chromatography yielding nearly 15 mg (~20 w% of the sample) white powdered by-product. Several NMR experiments including HH-COSY, HC-HSQC, HC-HMBC and NOE spectra were performed to identify the structure as pentahydroxyscirpene (PHS, Figure 1).

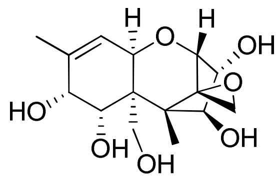

Figure 1. 3α,4β,7α,8α,15-Pentahydroxyscirp-9-ene (PHS)

To elucidate the origin of the compound as well as to ensure that it is not an artefact or degradation product, the whole fermentation process using the same *Fusarium* strain was repeated on rice for four weeks. Average concentrations of a biological triplicate of 1.38 ± 0.07 g/kg for NIV and 0.32 ± 0.04 g/kg for PHS were observed after three weeks of incubation.

An *in vitro* toxicity test, based on the inhibition of protein synthesis by trichothecenes, was performed to determine the toxicity of PHS. Translation of a firefly luciferase reporter mRNA by wheat ribosomes was suppressed to 50% (IC_{50}) at 0.5 µM NIV and 1.0 µM PHS. In comparison, the IC_{50} value of deoxynivalenol (DON) was also 1.0 µM. We conclude that the potency of PHS to inhibit protein biosynthesis is very similar to that of DON.

Infant exposure to mycotoxins with breast milk: Current state of knowledge

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An exposure of newborns and infants to toxic contaminants is of concern, since the developing organism may be more susceptible to adverse effects than that of adults [1]. Foods ingested by nursing mothers may have an impact on their babies, since many dietary contaminants, including certain mycotoxins, are absorbed by the mother's organism and can be transferred to her milk.
Important mycotoxins in breast milk, i.e. aflatoxins (mainly AFM_1 and AFB_1) and ochratoxin A (OTA), have been studied most extensively in this regard. We have now reviewed published data on their occurrence and levels in human milk. The reported frequencies of detection and average concentrations of aflatoxins indicate that exposure of infants in Europe is low or negligeable, but can reach critical levels in Tropical Africa and some countries of the 'Middle East'. The implications for infants' health will be briefly discussed.
The only other mycotoxin for which levels in human breast milk have been evaluated is OTA, a nephrotoxic compound with carcinogenic properties. In samples from most European countries, the OTA levels range between 10 - 400 ng/L. However, in some countries with no enforced mycotoxin regulations, such as Egypt, Turkey and Sierra Leone, OTA levels in human milk can be more than 100-fold higher than those measured in Europe. Based on OTA concentrations in breast milk, one can calculate the daily exposure of the babies taking into account their milk consumption and adjusting for body weight to arrive at an OTA intake in ng/kg bw/day. Then, the OTA intake of nursed infants in some countries will clearly exceed the TDI values that have been set for adults [2].
A toxicological assessment has to consider also that infants can be exposed concurrently to more than one mycotoxin with breast milk, e.g. aflatoxins and OTA. Yet, there are considerable gaps in knowledge regarding the lactational transfer of other mycotoxins in humans. Animal data on carry-over provide useful orientation on the degree of transfer of mycotoxins into milk. Yet, metabolism and secretion of several mycotoxins into milk can differ between ruminants and monogastric animals. This results in uncertainties with regard to effiency of lactational transfer in humans and infant exposure. Whilst it is unlikely that human milk contains fumonisins, DON and related trichothecenes, this cannot be excluded for some other mycotoxins that are ingested by nursing mothers.

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Influence of nanomolar concentrations of Ochratoxin A on gene expression in primary human kidney cells

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Multiple nephrotoxic substances lead to tubule-interstitial nephritis, an inflammatory process characterized by fibrotic alterations of the kidney. Ochratoxin A (OTA) is a mycotoxin suspected to cause interstitial nephritis but the underlying changes of gene expression, especially those after prolonged exposure to low and relevant concentrations of OTA, are still unknown. By using pathway specific qPCR-arrays covering a set of 218 genes we show that 14 days exposure of human primary proximal tubule cells (RPTEC) to 10 nM OTA influences the expression of genes that are related to inflammation (e.g. TNF-alpha, lymphotoxin-alpha (LTA)), malignant transformation (e.g. hypermethylated in cancer (HIC1), matrix metallopeptidase 10 (MMP10)) and epithelial-to-mesenchymal transition (EMT) (e.g. fibronectin (FN1), plasminogen activator inhibitor type 1 (SERPINE1)). WNT1 inducible signaling protein 1 (WISP1), an oncogenic and profibrotic growth factor, turned out to be the gene with the strongest upregulation. Its expression, as well as that of TNF-alpha, as an important inflammatory mediator, was investigated in further detail in RPTEC and in comparison in primary normal human lung fibroblasts (NHLF) and human embryonic kidney cells (HEK293). OTA potently increases WISP1 and TNF-alpha expression in RPTEC and HEK293, but not in NHLF, which is a hint for the cell-type and tissue-specific effect of OTA. Inhibition of ERK1/2 activation reverses the effect of OTA on WISP1 and TNF-alpha expression, whereas other signaling pathways, such as Wnt-signaling, seem to play no role. Our results demonstrate that a long-term exposure of human kidney cells with an OTA concentration expectable in human renal tissue leads to pathogenetic relevant alteration in the expression of genes involved in inflammatory, fibrotic and oncogenic processes. In particular WISP1 and TNF-alpha in an ERK1/2 dependent manner. Thereby OTA has the potential to initiate or support the development of interstitial nephritis.

Coexposure of Human Kidney Cells to Ochratoxin A and Fumonisin B_1 Stimulates Proliferation and a Synergistic Increase in Mitogen-Activated Protein Kinase.

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Fumonisin B_1 (FB_1) and ochratoxin A (OTA) are mycotoxins classified as possible human carcinogens (IARC, 1993; Pfohl-Leszkowicz & Manderville 2012). They co-exist in human food products and have been shown to act synergistically in stimulating nephrotoxicity in pigs (Diaz et al, 2001; Pfohl-Leszkowic & Manderville, 2007). The goal of the present study was to determine the combined impact of the toxins in cultured human kidney cells (HK2). Parameters common to both FB_1 and OTA (cytotoxicity and activation of mitogen-activated protein kinase, MAPK), and features characteristic of the individual mycotoxins (DNA adduction by OTA and release of arachidonic acid (AA) by FB_1) were measured. Treatment of HK2 cells with the combined toxin mixture (FB_1 + OTA) was found to stimulate cell proliferation. Under analogous conditions FB_1 was weakly cytotoxic while OTA showed no impact on cell viability. The conditions for cell proliferation by (FB_1 + OTA) were then examined for DNA adduction, release of AA and activation of MAPK. OTA-mediated DNA adduction, as measured by ^{32}P-postlabeling, occurred at a quicker rate in the presence of FB_1, with maximum levels being detected following 2 h incubation; with OTA alone adducts were not observed until 7 h incubation. Release of AA by FB_1 was inhibited by OTA, although levels of AA, prostaglandins (PGs) and leukotrienes (LTs) were higher for the combined toxin mixture than for OTA alone. Western blot analysis for MAPK activation showed the mycotoxins to synergistically enhance MAPK activity. Our data suggests that stronger nephrotoxic damages to human kidney are likely from combined exposure of (FB_1 and OTA) versus the individual mycotoxins, as resistance to apoptosis and stimulation of cell proliferation are key events in the development of tumors.

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Identification of genotoxic impact compounds in complex extracts of *Alternaria alternata* infested rice

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Fungi of the genus *Alternaria* are ubiquitously present in the environment, being part of the natural microflora in soils. They are saprophyts as well as plant pathogens, able to infect growing plants in the field. Furthermore, due to their ability to grow even at low temperatures, *Alternaria spp*. play a significant role in post-harvest decay during refrigerated transport of fruit and vegetables, but also stored grain and cereals, and lead to considerable economic losses. Over 70 secondary metabolites of *Alternaria* have been described, most of them acting as plant-toxins, some being toxic to humans and animals. In this context alternariol (AOH), alternariol monomethyl ether (AME), altenuene (ALT), tenuazonic acid (TeA) and altertoxin I (ATX I) are usually referred to as the main *Alternaria* mycotoxins with relevance for humans. In the present study we addressed the question whether these mycotoxins contribute substantially to the genotoxic properties of complex *Alternaria* toxin mixtures.
Four readily available strains of *Alternaria alternata* were cultivated on moist, autoclaved rice and extracts prepared from the cultures. These extracts showed strain specific mycotoxin profiles with high levels of the "main" *Alternaria* toxins alternariol (AOH), alternariol monomethyl ether (AME) and tenuazonic acid (TeA). Up to 690 mg AOH /kg rice culture were found with *A. alternata* DSM 12633 and up to 4.7 g TeA /kg rice culture with *A. alternata* DSM 62010. These extracts exhibited DNA strand breaking potential in human colon carcinoma cells HT29 in the comet assay, far greater than what can be explained by their content in AOH, AME or TeA. Using toxicity-guided fractionation, three HPLC peaks were found to cause the DNA damage, one of which was identified as altertoxin II (ATX II), a toxin described as potently mutagenic to *Salmonella typhimurium* in the Ames test. Incubation of HT29 cells with ATX II significantly increased the rate of DNA strand breaks at concentrations of 0.1 µM and higher and induced FPG sensitive sites to the DNA at concentrations of 0.05 µM ATX II and higher, whereas the highest incubation concentration was limited to 1 µM ATX II in order to prevent artifacts by cytotoxicity. The proliferation of HT29 cells, measured with the sulforhodamine B (SRB) assay, was significantly inhibited by ATX II in the same concentration range, beginning with 0.05 µM ATX II. This is in accordance with the observed cell cycle arrest of HT29 cells in G_0/G_1-phase by ATX II after 24 hours. However, no indication was found for oxidative stress or ROS formation by ATX II that would explain the significant induction of FPG-sensitive sites to the DNA, an effect that is not seen with AOH, AME or TeA.
Taken together, ATX-II was found to play a crucial role for the genotoxicity of complex extracts isolated from infested rice, whereas the AOH, AME and TeA appear to be of minor importance.

Reactivity of the mycotoxin patulin activated by glutathione: A putative mechanism of adduct formation with DNA bases

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As a frequent contaminant in moldy apples and apple products, the mycotoxin patulin (PAT) has shown mutagenic potential in cultured mammalian cells at concentrations which are equivalent to those found in marketable foods. This fact is in contrast with the assumption that the intracellular excess of glutathione (GSH) ascertains the complete inactivation of small reactive electrophiles such as PAT. Although PAT reacts readily with GSH, previous studies showed that co-incubation of PAT with model thiols and amine compounds increased the reactivity of PAT towards amines forming mixed-type adducts. Thus, we hypothesise that the potential to react with DNA bases after being activated by GSH might contribute to the mutagenicity of PAT.
Thus, cell-free reactions of PAT with DNA bases in presence and absence of equimolar concentrations of GSH were carried out. Liquid chromatography coupled with electrospray ionization tandem mass spectrometry was applied for identification and structure elucidation of putative adducts. Besides already known PAT-GSH adducts, several PAT-DNA base adducts were detected both in presence and absence of GSH. In addition, with each of the DNA bases one product exhibiting a mass to charge ratio and fragmentation pattern identifying a mixed PAT-GSH-DNA base adduct was observed. Moreover, one hitherto unknown PAT-GSH adduct was identified. Since its fragmentation pattern correlated with the PAT-GSH-DNA base adducts before the linkage to the DNA base, it might represent the precursor of the mixed adducts, suggesting the activation of PAT by reaction with GSH. Based on the fragment ions of adducts formed with GSH and by chemical modifications of adducts, we propose a structure of the mixed PAT-GSH-DNA base adducts and the putative mechanism of their formation.
Thus, the present study demonstrates the reactivity of PAT towards DNA bases and the participation of GSH in adduct formation. These findings are in contrast with the assumption that the conjugation of the major intracellular nucleophile GSH induces the complete inactivation of PAT. In fact, the formation of a reactive PAT-GSH monoadduct and its potential to associate with DNA bases could contribute to the mutagenicity of PAT.

Species specific glucuronidation of T-2 toxin by liver microsomes

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The trichothecenes T-2 toxin (T-2) and HT-2 toxin (HT-2) are produced by several *Fusarium* fungi and can be found in cereals like oat, corn or wheat. Several toxic effects have been described for T-2 and HT-2 and the current literature is summarized in a recent EFSA report (1).

In the course of various *in vitro* and *in vivo* studies numerous metabolites of T-2 were identified. T-2 is modified by typical phase I reactions like hydrolysis, hydroxylation or deepoxidation. The deacetylation product HT-2 was found to be one of the major metabolites of T-2. However, T-2 and its metabolites can also be conjugated in typical phase II reactions to polar compounds to form glucuronides, which can be excreted more easily (2,3).

These glucuronidation reactions are catalyzed by UDP-glucuronosyltransferases (UGTs), which transfer the glucuronide moiety from uridine 5'-diphosphoglucuronic acid (UDPGA) to the xenobiotic substance. Since UGTs are located in the endoplasmic reticulum of cells, liver microsomes can be used as a source for these enzymes.

Using liver microsomes glucuronides of T-2 and HT-2 were synthesized. After clean-up, glucuronide structures were elucidated by nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry. Moreover microsomes prepared from livers of different species served as an *in vitro* model to study differences in the glucuronidation. Distinct variations could be observed depending on the animal species.

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Metabolism of zearalenone in piglet liver *in vitro*

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Zearalenone (ZEN) is a ß-resorcylic acid lactone produced by *Fusarium* species infesting mostly corn but also other grains. It has a rather low acute toxicity, and the biological activity of this mycotoxin is dominated by it estrogenicity, resulting in severe effects on the reproductive system of farm animals, in particular pigs. Although ZEN has been shown to undergo reductive, oxidative and conjugative metabolism in various mammalian species, the capability of piglets to metabolize ZEN is still unknown.

Microsomes and cytosol were prepared from the liver of one untreated piglet, and the phase I metabolism of ZEN by cytochrome P450 (CYP) and hydroxysteroid dehydrogenase (HSD), as well as conjugation with glucuronic acid and sulfate were studied. Glucuronidation of ZEN was observed at a rate of 210 nmol/min/g liver, leading to the 14-*O*-glucuronide and 16-*O*-glucuronide at a ratio of 99:1. Sulfate conjugation with liver cytosol was much lower, and only small amounts of the 14-*O*-sulfate were detectable with LC-MS. Both microsomes and cytosol reduced ZEN to alpha-ZEL and beta-ZEL at a ratio of 3:1 and a rate of 27 nmol/min/g liver. The same activity of HSD was found for the dehydrogenation of alpha-ZEL to ZEN. CYP-mediated oxidation with microsomes gave rise to the catechols 15-HO-ZEN and 13-HO-ZEN as the major oxidative metabolites at a ratio of 3:1 with an activity of 5 nmol/min/g liver. 8-HO-ZEN and small amounts of other aliphatic hydroxylation products were also formed.

In order to study the effect of long-term exposure to ZEN and deoxynivalenol (DON) on the metabolic activity of piglet liver, two groups of piglets were fed maize contaminated with either background levels of 0.005 mg/kg ZEN and 0.03 mg/kg DON (control animals) or with 0.3 mg/kg ZEN and 4.5 mg/kg for 29 days. In hepatic microsomes from the piglets with high exposure, the total amount of CYP was about 25% lower than in the control group. The activity of CYP was reduced accordingly, but no alteration of the pattern of oxidative metabolites and no effect on the glucuronidation of ZEN were observed.

In conclusion, our study has shown that piglet liver is capable of oxidative and reductive metabolism, as well as glucuronidation of ZEN. In contrast, conjugation with sulfate, which is a major phase II pathway in other species, is low in piglets. Exposure to ZEN and DON causes a selective decrease of CYP activity in piglet liver.

Microarray analysis of differentially regulated genes in the porcine intestinal cell line IPEC-J2 upon site directed exposure to deoxynivalenol

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Background and aims: The cereal-contaminating, *Fusarium*-derived mycotoxin deoxynivalenol (DON) causes health risks for animals and humans. It is known to be rapidly absorbed through the intestinal barrier into the animal's body during the gut passage. The polarised intestinal enterocytes are exposed to the mycotoxin from both directions, the apical, mucus protected luminal side, as well as the basolateral, blood stream supported site. In the present study, we analysed the mechanisms of apical and basolateral DON toxicity reflected on the gene expression profile of porcine intestinal epithelial cells (IPEC-J2).
Methods: The intestinal porcine epithelial cell line IPEC-J2 was cultured until confluence (transepithelial electrical resistance, TEER>1kOhm) on permeable membrane inserts, serving as polarised *in vitro* model for the intestinal barrier. The effect on IPEC's gene expression, exposed for 72 h to either low (200 ng/ml) or high (2000 ng/ml) DON concentrations from either apical or basolateral side, were examined by GeneChip® Porcine Genome Array of Affymetrix and evaluated by the bioinformatic research tool DAVID. A range of genes from various biochemical pathways was selected and their gene expression analysed by qPCR.
Results: The analysis of the generated gene expression profile revealed that low basolateral DON application (200 ng/ml) triggered 10 times more gene transcripts than the corresponding apical exposure (2539 versus 267 changed genes). The challenged cell layer stayed completely intact, as measured by TEER. Most changes were seen with 2000 ng/ml DON from apical side (3589 genes), while its basolateral counterpart still showed 669 changed genes although the cell layer was severely compromised. Examination of the regulated genes with DAVID revealed several groups of biochemical pathways, modulated by concentration and site direction of DON application. Representative genes for biochemical pathways of cellular metabolism (e.g. succinate dehydrogenase), information processing (e.g. ribosomal protein RPL10a) and structural design (e.g. claudine-3) were further quantified by qPCR and confirmed the microarray data.
Conclusions: Here we show that the application route of the mycotoxin deoxynivalenol on the intestinal epithelial barrier triggers different gene response profiles. On the transcriptional level the cells exhibited the highest susceptibility towards basolateral challenge of the low DON concentration, while physiologically no changes of the epithelial layer were detectable (Diesing et al., 2011). In light of our results, we suggest to take the impact of the application route into account when investigating toxicological substances.

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The genotoxic effect of deoxynivalenol in broiler chickens

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Deoxynivalenol (DON) is unavoidable contaminant in foods and feeds and is a major problem all over the world. It is suggested that DON induces free radical production that may be one of the mechanisms causing membrane and DNA damage (1). Moreover, it was shown that DON caused DNA fragmentation in chicken spleen leukocytes (2). Therefore the effect of feeding of DON contaminated diet on the blood lymphocyte DNA damage in broiler chickens was investigated.

Twenty 1-d-old broiler chicks were randomly divided into 2 groups. The control group was fed non-contaminated diet and a second group was fed a diet artificially contaminated with 10 mg DON/kg diet. The basal diet was formulated based on wheat, soybean meal, maize, rapeseed oil, and a premix with vitamins, minerals, amino acids, salt, and Mono-calcium phosphate. Nutrient concentrations were formulated to meet or exceed minimum requirements for broilers according to the National Research Council (3). The diet for the control birds was the basal diet without addition of DON. Feed and water were provided *ad libitum*.On day 35, blood was collected in heparinised tubes from 8 chicks per group. After lymphocyte isolation, the degree of blood lymphocyte DNA damage was measured by Comet assay for cytotoxicity testing. This study was approved by the institutional ethics committee and the national authority according to the law for Animal Experiments, Tierversuchsgesetz—TVG BMWF, GZ-68.205/0032-II/10b/2010.

Dietary deoxynivalenol increased the amount of DNA damage (32 %) of chicken blood lymphocytes compared with the control group (21 %) as indicated in the portion of DNA in the tail of comet.

From this study, it can be concluded that contamination of broiler diet with deoxynivalenol induced blood lymphocyte DNA damage. DON affects primarily the rapidly proliferating cells of the immune system as a result of inhibition of protein synthesis. DON should further be considered as a genotoxic hazard factor in risk assessments.

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Chronic effects of deoxynivalenol on plasma cytokines and vaccine response of broiler chickens

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An important effect of the trichothecene mycotoxins is the impairment of immune function. Studies on immunotoxicity of deoxynivalenol (DON) have been mainly conducted in mouse models. In addition, cellular and molecular mechanisms for immune modulation by DON are contradictory (1). Nevertheless DON was shown to suppress the antibody response to infectious bronchitis vaccine (IBV) in chickens (2). In the present study, the effects of dietary DON on the concentrations of tumor necrosis factor alpha (TNF-α) and interleukin 8 (IL-8) in plasma of broiler chickens were quantified. The immune response of chickens to infectious bursal disease (IBD) after DON exposure was also investigated. A total of 20 day-old broiler chicks were obtained from a commercial hatchery and randomly divided into 2 groups: 1) control group, 2) DON-treated group (10 mg/kg). The birds were housed in battery cages for 5 wk. The diet for the control birds was the basal diet without addition of DON. At day 35 blood samples were collected in heparinized tubes. The concentrations for tumor necrosis factor alpha (TNF-α) and interleukin 8 (IL-8) were quantified in plasma, using commercially available ELISA test kits (Antibodies-online.com). Moreover, birds were vaccinated at the first day of life and 14 days of age via drinking water. The antibody titer for infectious bursal disease (IBD) was measured by the anti-IB antibody ELISA kit. DON reduced TNF-α concentration. TNF-α decreased approximately two folds in DON-fed birds compared to control birds (17.2±5.5μg/ml and 30.7±3.7 μg/ml, respectively). These results indicate that DON reduced the level of TNF-α in the plasma of broiler chickens. However, DON did not modify the plasma levels of IL-8 (3.24±0.01 μg/ml and 3.23±0.02 μg/ml in control and DON fed birds respectively).The results showed also that the feeding of DON-contaminated diets decreased serum antibody titer against IBD (1972 IU) at 10-d-old compared with controls (2599 IU). Moreover, IBD titer was also reduced at 35 days old in DON group (79 IU) compared with control group (412 IU). These results indicate that immunotoxic effects of DON in broiler diets are rather based on changes in plasma TNF-α and decreased the immune response against disease. In conclusion, DON had adverse effects on immune function of broilers. Further experiments are needed to clarify the complexity of DON immunotoxicity in broilers.

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Deoxynivalenol predisposes for the development of necrotic enteritis in broilers

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Clostridium perfringens induced subclinical necrotic enteritis (NE) causes important economic losses in the broiler industry. The *Fusarium* mycotoxin deoxynivalenol (DON) may affect the intestinal epithelial integrity, subsequently inducing protein leakage into the intestinal lumen. The objective of this study was to examine whether DON at contamination levels below the maximum guidance level in poultry feed is a predisposing factor for NE in broilers.
In this study we used a highly reproducible *in vivo* infection model mimicking subclinical NE (Gholamiandehkordi et al., 2007). A total of 360 one-day-old Ross 308 broilers were randomly divided into four groups of three replicates with 30 birds per replica. All birds were fed a starter diet during the first eight days of the experiment, subsequently a grower diet for eight days, followed by a finisher diet until euthanasia. Throughout the entire experiment, groups 1 and 4 received a blank diet while groups 2 and 3 received a diet experimentally contaminated with DON. All birds in group 1 and 2 were challenged orally with *C. perfringens* strain 56 containing approximately 4×10^8 cfu/ml for four consecutive days starting at day 17. The remaining groups received sterile medium.
The blank feed contained DON at 75 ± 22 µg/kg (starter), 83 ± 24 µg/kg (grower) and 100 ± 29 µg/kg (finisher). The contaminated feed contained DON at 3761 ± 1100 µg/kg (starter), 4281 ± 1300 µg/kg (grower) and 4384 ± 1300 µg/kg (finisher).
At 1, 2 or 3 days after the final challenge with *C. perfringens*, chickens were euthanized and scored macroscopically for intestinal NE lesions. Chickens that received DON and *C. perfringens* had significantly (alpha=0.05, P<0.001) more lesions than chickens that received only *C. perfringens*, with 46.6% and 19.5% of chickens positive for NE lesions, respectively. In non-inoculated groups no NE lesions were present.
In conclusion, the presence of DON in the feed in concentrations lower than the maximum guidance level of 5000 µg/kg is a predisposing factor for the development of NE in broilers.

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Acknowledgements
This work was financially supported by BIOMIN GmbH, Austria.

Effect of increasing concentrations of deoxynivalenol in diets of laying hens of different genetic background and roosters on reproductive performance and health of the hatched chicks

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Deoxynivalenol (DON) is a well known *Fusarium* mycotoxin that frequently infects wheat, corn, and other grains. The diversion of DON contaminated and suspicious cereal batches into poultry feeding due to the high tolerance of poultry to DON are expected to result in higher exposure of this animal category. Moreover, no data on the effect of hen's breed on the sensitivity towards DON are available.

Methods: A total of 216, 23 weeks old laying hens (108 Lohmann brown, LB, and LSL, respectively) were assigned to a feeding trial with increasing diet concentrations of DON (0.0, 3.3, 10.0 mg/kg), resulting in 6 experimental groups of 36 hens each. In addition, 24 adult roosters were divided into three groups (8 roosters each) and fed similar diets. All birds were caged individually and had *ad libitum* access to feed and water. Beginning at the 30th and 60th week of life eggs were collected three times for hatching; hen feeding groups were divided into three insemination groups (12 hens each) and then inseminated with mixed semen from roosters of one group after an insemination design providing the insemination of each hen with semen from each rooster group. At the 60th week of life and after hatching, 30 chicks of selected groups (control hens x control roosters; highest DON hens x highest DON roosters) were weighed and killed. Blood was collected into heparinised tubes. Liver, spleen, gizzard, heart, yolk sack, and intestine were dissected and weighed. All data were evaluated by ANOVA; means were compared by Fischer-LSD test ($p<0.05$).

Results: Hatchability was adversely affected by the presence of DON in hen's feed while the hatchability of the LSL eggs was significantly higher than of the LB eggs (fig.1). Moreover, a significant interaction between DON in hen's diet and breed of hens was noticed on fertility (the percentage of fertile eggs of all laid eggs) as it was decreased in the eggs of the 10-LB and increased in the eggs of the 10-LSL. Body weight was increased significantly in the chicks hatched from the eggs of 10-LSL compared with the control group (fig.2).

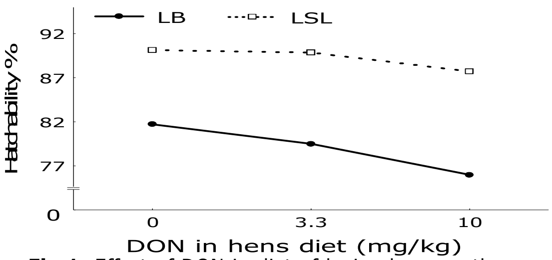

Fig.1: Effect of DON in diet of laying hens on the hatchability % (means, n=108, p<0.05).

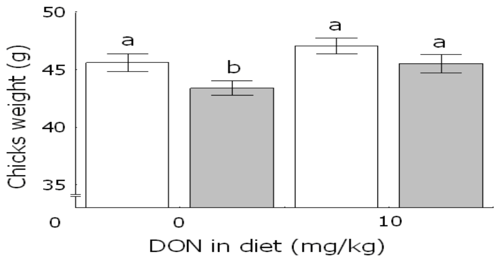

Fig.2: Effect of DON in diets of hens and roosters on the body weight of the hatched chicks. (Means ± SE, n=30). a,b different Letters show significant differences, (p<0.05), □ LSL, □ LB.

Conclusion: The current results indicate a negative impact of DON in hen's diet on hatchability. In addition the breed of hens seems to be ab additional factor influencing the effect of DON on fertility.

Time dependent effects of graded levels of *Fusarium* toxin contaminated maize grain in diets on female piglets

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The *Fusarium* mycotoxins deoxynivalenol (DON) and zearalenone (ZEN) are prevalent contaminants of animal feed and are often detected in the same samples. Since pigs are regarded as the most sensitive species in livestock production, guidance values for piglet diets of 0.9mg DON/kg and 0.1mg ZEN/kg should not be exceeded (The Commission of the European Communities, 2006). High concentrations of DON were reported to affect performance and immune system, whereas ZEN possesses oestrogenic properties that may induce hyperestrogenism. Dietary ZEN is subjected to entero-hepatic circulation and may thus be accumulated in bile fluid. Therefore, even low dietary concentrations may result in hyperestrogenism due to prolonged exposure. The aim of the present study was to investigate the progression of adverse effects of both toxins in dependence on increasing dietary concentrations and time of exposure.
120 female cross-bred piglets (7.85±3.3kg body weight [BW]) were allocated to 20 pens (6 piglets per pen) and assigned to 5 groups receiving diets with increasing percentages of contaminated maize grain (0, 1.25, 2.5, 5 and 10%), resulting in 0.01, 0.05, 0.08, 0.17 and 0.29 mg ZEN/kg and 0.03, 0.59, 1.27, 2.01 and 4.52 mg DON/kg (Groups 1-5, respectively). The animals had free access to feed and water. Performance parameters and individual vulva widths were recorded weekly. After feeding periods of 1, 3, 8, 15, 22 and 29 days a total of 20 piglets at each time point (4 animals per group) were subjected to blood sampling for serum analyses and necropsy. The obtained data were analysed using the PROC MIXED procedure of the software package SAS version 9.1 considering experimental day, diet and their interaction as fixed effects.
Diet had a significant effect on mean daily feed intake (p=0.039), which was reduced by more than 20% in group 5 receiving 10% contaminated maize. The evaluation of the different experimental weeks revealed significantly decreased feed intakes exclusively in the first week for group 5 compared to groups 1 and 2. Mean daily BW gain was affected by diet (p=0.025), but BW did not differ among the experimental groups (p>0.05). Though not significant, vulva width corrected for BW was highest in group 5 and group differences increased with progressing experimental time. Relative uterus weights tended to be influenced by dietary treatment (p=0.08) and increased nominally in group 5. Serum clinical chemical parameters were found to be mainly within the reference range and did not indicate considerable differences between the groups.
The highest investigated contaminations (3- to 5-fold higher than the guidance values) may affect performance parameters such as mean daily feed intake and body weight gain and induce a slight hyperestrogenism which became more pronounced with increasing time of exposure.

Reference

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Effects of Fusarium mycotoxins in rations with different concentrate proportions on insulin sensitivity in lactating dairy cows

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Background: The fusarium toxin Deoxynivalenol (DON) is a frequently found contaminant in dairy rations. Although ruminants are less sensitive to DON than monogastrics due to detoxification during ruminal microbial fermentation, this process may be less effective when high amounts of concentrates are fed. The cytotoxicity of DON is explaind by the ribotoxic effects of DON potentially influencing translation and transcription of genes. Reduced insulin sensitivity is assumed to contribute to metabolic disorders in high yielding dairy cows. Thus, the objective of the study was to investigate the effect of long term DON exposure on insulin sensitivity and transcription of muscular and hepatic glucose transporters (SLC2A) and insulin receptors (IR) in lactating cows fed diets with different amounts of concentrate.
Materials and methods: In period 1 27 German Holstein cows were assigned to two groups fed a control (Con) or mycotoxin-contaminated diet with 50% concentrate (Myc, 5.3mg/kg DM DON and 112.7µg/kg DM Zearalenon) for 11 weeks. In the following 16 weeks (period 2) each group was further divided and fed either a diet containing 30% or 60% concentrate (Con30, Con60, Myc30:4.4mg/kg DM DON and 73.8µg/kg DM Zearalenon, Myc60, 4.6mg/kg DM DON and 72.5 µg/kg DM Zearalenon). Blood samples were taken in week (w) 0, 4, 8, 15, 21, 27 and the Revised Quantitative Insulin Sensitivity Check Index (RQUICKI) was calculated from serum glucose, non-esterified fatty acids (NEFA), and insulin concentrations. Muscular (*M. semitendinosus* or *semimembranosus*) and hepatic biopsy samples were taken on w 0, 15 and 27. Transcription level of SLC2A (isoform 1 and 4 in muscle, isoform 1 and 2 in liver) and IR isoform A, B in both tissues was measured with real-time RT-PCR. Statistical analysis was performed using the mixed model procedure for repeated measures of SAS with dietary DON and concentrate, sampling week, and interactions as fixed effects.
Results: In period 1 no DON effects were detected. In period 2 DON groups showed higher insulin levels ($p<0.01$) and lower insulin sensitivities (RQUICKI; $p<0.05$) than controls. Compared to controls DON exposure increased muscular mRNA expression of SLC2A1 in w 15 ($p<0.05$) while lower expressions were observed for both IR isoforms in w 27 ($p<0.01$). Only in the DON group with high dietary concentrate proportion SLC2A4 was significantly ($p<0.05$) reduced in w 27 compared to controls in w 27. The level of SLC2A2 in liver was lower in animals of DON groups in week 27 ($p<0.01$). No effects were detected for hepatic SLC2A1 and IRs in liver. Significant interactions between DON and dietary concentrate were found only in muscular SLC2A4 and IRB.
Conclusion: Long term exposure to dietary DON contaminated diets may induce reduced insulin sensitivity in lactating dairy cows especially when high dietary concentrate amounts are fed.

Adverse effects of food-borne deoxynivalenol (DON) exposure on carp (*Cyprinus carpio L.*)

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Contamination of fish feeds with *Fusarium* toxins is a major problem due to increased usage of cereal-based ingredients. Deoxynivalenol (DON) is a secondary metabolite of these fungi contaminating corn and cereal cultures in agricultural used regions all over the world. Mycotoxin-contaminated fish food has already been shown to induce changes in swimming behaviour and to impair growth of fish, but DON has not yet been evaluated for its toxic potential in fish in detail. This would be necessary since a general recommendation on guidance values of DON in complete feeding stuffs and complementary feeds of 5 mg DON per kg (at 88 % dry matter) was established by the European Commission (2006/576/EC). Thus, in *in vivo* experiments carp were fed concentrations of 350 to 950 □g DON per kg feed for 4 weeks to show possible effects of this mycotoxin at concentrations below the recommended minimum values. Weight gain and condition factors of fish were not influenced after 4 weeks of DON feeding. However, differences in nutritional status and fitness have been observed in DON-treated fish. In addition, damages to organs (determined by means of lipid peroxidation in organs) and histological changes in tissues were noted. Immunological studies on primary kidney cell cultures of experimentally intoxicated fish indicate modulation of immune responses at high DON concentration in the feed. From these experiments it can be concluded that the mycotoxin DON exerts several detrimental effects on fish.

Effects of a deoxynivalenol (DON) contaminated diet on serum DON concentrations, feed intake and health status in horses

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Information on the effects of deoxynivalenol (DON) in equines is limited and controversial. The aim of the study was to investigate the effects of DON exposure on feed intake and health status in horses.
12 healthy geldings were randomly assigned to one of the following treatments (n=4-5/treatment): control; 50 µg or 75 µg DON/kg BW. DON was provided via contaminated wheat (9.6-18.6 mg/kg) for 21 d. The total wheat intake was adjusted to 4 kg for all horses, and comprised different proportions of contaminated wheat depending on the treatment group. Two kilograms of wheat plus 0.85 kg haylage/100kg BW (ryegrass haylage, DM: 78 %) was provided in the morning and in the evening. Blood samples were collected at predetermined times for routine haematology and to determine circulating DON, de-epoxy-deoxynivalenol (DOM-1) and serum globulins concentrations as well as serum liver enzyme activities (aspartate aminotransferase, gamma glutamyl transferase, glutamate dehydrogenase, bile acids). After chronic DON exposure (21. d) the proliferation response of peripheral blood mononuclear cells (PBMC) was assessed *ex vivo* using MTT assay. After a wash-out period of 6 weeks, free choice feeding was undertaken with uncontaminated and contaminated wheat as well as corn.
Wheat intake was significantly influenced by the level of DON exposure of 75 µg DON/kg BW, decreasing with the provision of 75 µg DON/kg BW. The negative impact on feed intake of DON contaminated wheat was confirmed in the free choice feeding study by an immediate total refusal of DON contaminated wheat or corn. There was no effect on haylage intake, blood cell counts, liver enzyme activities or serum globulins. Serum DON concentrations increased with increasing DON intake in a linear manner. Negligible DOM-1 concentrations were only detected in serum samples on day 21 of DON exposure suggesting that DON was absorbed in the small intestine in its non-metabolized form to a large extent and/or poorly degraded to the less toxic metabolite DOM-1 in the hindgut. *Ex vivo* proliferation responses by non stimulated or Con A stimulated PBMC were not significantly by DON exposure.
The serum DON concentration increased in a dose-response-related manner likely to swine, but beside that it seems that the horse tolerates relative high DON concentrations in the ration without adverse effects on health status. However, during the 21 d of DON intake, results reflect an aversion response, possibly mediated through a cognitive effect. However, the immediate refusal to eat the DON contaminated wheat or corn during the free choice feeding trial suggests that the impact on feed intake might be due to sensory changes within the DON infected wheat rather than post ingestion central effects. In conclusion, horses seemed to be less or at most similar susceptible to DON exposure than pigs, therefore the benchmark of 1 mg DON/kg in grains or concentrates for horses seemed to be safe.

In Vitro And *In Vivo* Safety Testing Of Mycotoxin Detoxifying Agents

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Adding mycotoxin detoxifying agents to feed contaminated with mycotoxins is commonly used and seems to be the most promising way of counteracting the harmful effects of mycotoxins. The European Food Safety Authority (EFSA) stipulates that the interaction of these products with oral absorption of drugs and nutrients should be investigated. The binding of drugs by these products in the gastro-intestinal tract could lead to subtherapeutic plasma and tissue concentrations and enhanced microbial resistance. Nevertheless, only few literature is available and no models are present to evaluate these possible interactions. Therefore, the goal of this study was to develop an *in vitro* and *in vivo* model for safety testing of these products. The interaction between bentonite and tylosin was investigated as a possible interaction was described previously. In addition, a commercially available detoxifying agent (gluco-mannan, GMA) was tested.
First, *in vitro* trials were carried out. Intestinal porcine epithelial cells (IPEC-J2) were cultivated on Transwell® cell culture inserts until differentiation (21 days). Tylosin (Tylan® Soluble, Elanco, Belgium) (20 µg/mL) was added to the basolateral compartment with or without addition of a mycotoxin detoxifying agent (bentonite or gluco-mannan (GMA), 1 mg/mL) for 48 h. To determine the safety of bentonite and GMA towards adsorption of tylosin, several parameters were evaluated: cellular viability by means of a neutral red assay, cellular integrity based on the trans-epithelial electrical resistance (TEER) and passage through the monolayer by quantification of tylosin in the apical compartment using LC-MS/MS. No differences were found in viability or TEER between groups, indicating that the tylosin, bentonite or GMA concentration applied was not cytotoxic. Quantification of tylosin in the apical compartment showed a significant lower passage of tylosin due to binding to bentonite. Apical tylosin concentrations of GMA treated inserts did not differ from controls.
Next, these findings were evaluated by an *in vivo* model. Tylosin (24 mg/kg BW) was administered as a single intra-crop bolus to broiler chickens (n=8), with or without a mycotoxin detoxifying agent (bentonite or GMA, 1 mg/kg BW). Blood was withdrawn at several time points after bolus administration and plasma concentrations of tylosin were determined. Plasma concentration-time profiles were evaluated and several pharmacokinetic parameters were compared to the control group in order to evaluate the interaction between tylosin and bentonite/GMA. Plasma concentrations of tylosin were significantly lower after bolus administration of tylosin and bentonite, compared to the control group. No interaction between tylosin and GMA was found. These *in vivo* results confirm our *in vitro* findings.

Acknowledgments
This work was supported by the Agency for Innovation by Science and Technology in Flanders (IWT, Brussels, Belgium, SB n°101301)

Effect of T-2 toxin and a yeast-extract feed additive on the oral bioavailability of chlortetracycline in pigs

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Yeast-extracts derived from *Saccharomyces cerevisiae* can be used as feed additive to improve animal health by promoting intestinal flora and by stimulating the immune system (Patterson, 2005). The impact of such feed additives on intestinal absorption of antibiotics has however not been described yet. T-2 toxin is an emerging *Fusarium* mycotoxin, that can be present as feed contaminant and for which it is known it disturbs the intestinal integrity. It was therefore the aim of the present study to determine the pharmacokinetic (PK) parameters of orally administered chlortetracycline in pigs after intake of 100 µg/kg T-2 toxin contaminated feed whether or not supplemented with a commercially available yeast-extract feed additive. Twenty-four 9-week-old pigs (Landrace) were randomized into a control group (n = 6) and three experimental groups (each n = 6). After an acclimatization period of one week, the groups were given respectively blank feed (control group), blank feed supplemented with the additive (Alphamune®, Pfizer, Belgium), feed experimentally contaminated with 111 µg T-2/kg feed and T-2 contaminated feed supplemented with the additive. Two hours after the start of intake of the respective feeds, the animals were given a single oral intragastric bolus of chlortetracycline at 20 mg/kg BW (bolus 1) (Aurofac®, Alpharma, Belgium). Blood samples were taken at 30′, 1h, 1.30h, 2h, 3h, 4h, 6h, 8h, 12h and 24h post administration. Next, after 21 days of intake of the experimental feeds, the animals were given a second oral bolus of chlortetracycline (20 mg/kg BW) (bolus 2). Blood samples were collected as described for bolus 1. Plasma concentrations of chlortetracycline were determined using an in-house validated HPLC-UV method. Pharmacokinetic parameters such as area under the plasma concentration-time curve ($AUC_{0\text{-}24h}$) were determined by means of the WinNonlin®, Version 6.2.0 (Pharsight Corporation, Mountain View, CA, USA) software program. After euthanizing the animals, samples of duodenum, jejunum and ileum were taken to determine villus length and crypt depth. Also the effect on the mucous layer was studied using a RT-PCR method for determination of mucin 1 and 2. Significant increased plasma concentrations of chlortetracycline compared to the control group were seen after intake of T-2 contaminated feed supplemented with the additive (bolus 1) and after intake of both T-2 contaminated and T-2 contaminated feed supplemented with the additive (bolus 2). More results will be presented at the congress.

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Acknowledgements

This work was supported by the Institute for the Promotion of Innovation by Science and Technology in Flanders (IWT-Vlaanderen), Brussels, Belgium (N° 070574).

Enzymatic hydrolysis of fumonisin B1 *in vivo*

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Introduction: Produced by a variety of *Fusarium* species, fumonisin B_1 (FB_1) is one of the most abundant mycotoxins in warm climate regions. Recent *in vitro* experiments showed that FB_1 can be hydrolysed to HFB_1 by the enzyme FumD (Heinl et al. 2010). Feeding experiments with piglets and rats reported strongly reduced toxicity of HFB_1 with respect to sphingolipid metabolism, neural tube defects and at hepatic and intestinal level (Voss et al. 2009, Grenier et al. 2012). The aim of this work was 1) to investigate the capability of different units of spray dried FumD mixed into feed to hydrolyse FB_1 in growing piglets and 2) to compare different biomarkers for assessing the extent of hydrolysis.
Experimental: To this end, growing piglets received feed contaminated with 30 mg/kg FB_1 containing different units of spray dried FumD (0.1, 1, 10, and 100 U/kg feed, n = 2 / unit) *ad libitum* for 2 weeks. Piglets receiving spray drying carrier in contaminated feed without FumD served as FB_1 positive control group (n = 8), piglets fed non contaminated feed plus spray drying carrier as negative control (n = 8). Once a week, faeces, serum and urine samples were collected and analysed for the concentrations of fumonisins (FB_1, $pHFB_{1a}$, $pHFB_{1b}$, HFB_1). Furthermore, the ratio of the sphingoid bases sphinganine (Sa) to sphingosine (So) and the ratio of their phosphates was determined. All analyses were carried out by LC-MS/MS after different sample preparation methods.
Results: Addition of 10 U/kg feed resulted in partial hydrolysis of FB_1, whereas addition of 100 U/kg achieved almost complete conversion of FB_1 to HFB_1. This was evidenced by lack of FB_1, but detection of HFB_1 in urine and faeces and by serum Sa/So and Sa-1-P/So-1-P ratios similar to those of the negative control group. Results for the following biomarkers corresponded nicely: Fumonisin concentrations in faeces, fumonisin concentrations in urine, and Sa/So and Sa-1-P/So-1-P ratios in serum.
Conclusion: Our results showed that the minimum dose of FumD required for complete gastrointestinal hydrolysis of 30 mg/kg FB_1 in feed is in the range of 10 to 100 U/kg. Several biomarkers were found to be suitable for monitoring *in vivo* fumonisin hydrolysis and detoxification. Feeding trials with larger experimental groups and narrower ranges of tested FumD concentrations are in progress.

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Degradation of deoxynivalenol by nematodes and collembolans in wheat straw depending on soil texture

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A microcosm-study under laboratory conditions was conducted to assess the interaction between soil fauna (*Aphelenchoides saprophilus,* Nematoda and *Folsomia candida,* Collembola) and the mycotoxin deoxynivalenol (DON) produced by *Fusarium* species. The objective was to investigate, if the introduced soil fauna is able to reduce the concentration of DON in wheat straw. Furthermore, the study aimed whether the degradation efficiency of the soil fauna is affected by different soil texture (sandy loam, silt loam, clay loam). Therefore, cylinder shaped microcosms were filled with 150 mg of soil and 200 mg of ground wheat straw (1.5 mm). Soil fauna was introduced in different combination into microcosms (single collembolan-, single nematode-, mixed- and a control treatment without animals). The microcosms were divided into 2 sets: one set received artificially DON-contaminated wheat straw, the other set received non-infected straw. After 2 and 4 weeks, samples of soil and straw were taken and soil fauna was counted. DON content was detected by using ELISA-method. Each treatment was replicated five times.
After 2 weeks DON was reduced in straw throughout all treatments, whereas the highest reduction was determined in the mixed treatment due to soil fauna interaction. After 4 weeks the degradation of DON in straw was even higher and again the highest reduction was found in the mixed treatment. In contrast to the abundance pattern of *A. saprophilus*, *F. canidida* showed a notable increase in the non-infected treatments after 2 and 4 weeks. The number of *A. saprophilus* increased mainly in the infected treatment. Furthermore the soil texture affected the degradation efficiency of the introduced soil fauna significantly. The DON concentration in the sandy- and silt loam treatments were reduced more efficiently compared to the clay loam treatment. We conclude that especially the interaction of nematodes and collembolans significantly contribute to the degradation of the mycotoxin DON in wheat straw as an ecosystem service.

Abstracts of posters

Multi mycotoxin analysis of feed by UPLC-MS/MS

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Mycotoxins are routinely determined by individual methods intended to a limited number of analytes. These methods usually include an imunoaffinity column cleanup which makes them expensive and time consuming. Moreover, their applicability to analysis of up-to-date monitored mycotoxins is limited. Multiresidue methods help us to overcome these inconvenience at mycotoxin analysis stated above. The method for the determination of 17 mycotoxins in feed based on the unbuffered QuEChERS method has been developed. The raw acetonitrile extract was diluted by deionized water and directly injected to an ultra performance liquid chromatograph coupled to a tandem mass spectrometer (UPLC-MS/MS). Chromatographic separation of the 17 analytes was performed by a gradient elution on a C18 phase column within 10 minutes. Mycotoxin detection was carried out in multi reaction monitoring (MR) mode and positive electrospray ionization. The present method has been validated for the determination of Deoxynivalenol, Nivalenol, HT-2 toxin, T-2 toxin, Ochratoxin A, Zearalenon, Aflatoxins B_1, B_2, G_1, G_2, Fumonisins B_1, B_2, Beauvericin and Enniatins A, A_1, B, B_1 in cereals and complete feedingstuffs. The validated method fulfilled criteria for residue analyses and requirements on detection and quantification limits for mycotoxin analysis in feed.

Validation data for a multi-toxin method for four different representative matrices

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In the recent years, LC-MS/MS based multi-analyte methodology has been successfully introduced into the field of mycotoxin analysis. Most of the related methods, however, still focus on those mycotoxins that are addressed by regulations and to some of their derivatives. In addition, many of the published methods focus on grain-based matrices such as maize or wheat, whereas application to other types of matrices has been hardly investigated.
In this work we present validation data for an LC-MS/MS protocol covering 320 fungal and bacterial metabolites for four different matrices: apple puree for infants (high water content), hazelnuts (high fat content), maize (high starch or protein content, low fat content) and green pepper (complex matrix). In case of the three former matrices, signal suppression/enhancement as well as recoveries of the extraction step were within the target range of 70-110% for more than 80% of the investigated analytes, whereas considerable matrix effects and extraction losses were observed for approximately half of all the analytes in pepper. The LOQs were below the regulatory limits with the exception of aflatoxins and ochratoxin A in baby food and aflatoxin M1 in milk. The accuracy of the method was further verified by the analysis of certified reference materials and by participation in ring trials. Z-scores were generally between -2 and 2 although the related samples derived from a broad variety of different matrices such as coffee, peanuts, baby food and animal feed.
Based on these results we conclude that quantitative analysis by LC-MS/MS based on a "dilute and shoot" approach is also feasible in case of non-grain based matrices.

Development and validation of an LC-MS/MS based method for the determination of deoxynivalenol, deoxynivalenol-3-glucoside and their metabolites in urine and faeces of rats

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Mycotoxin glucosides, formed by growing plants infected with toxigenic fungi, escape routine detection and have the potential to be reactivated in the digestive tract of mammals after hydrolysis to their parent toxins. Berthiller et al. (2011) showed that several lactic acid bacteria have the capability to hydrolyze deoxynivaleol-3-beta-D-glucoside (D3G) to deoxynivalenol (DON) *in-vitro*. Nevertheless, *in-vivo* studies about the adsorption, distribution, metabolism and excretion (ADME) are necessary to assess the potential health risk of D3G. Therefore, analytical methods which are sensitive and specific enough to detect DON, D3G and their metabolites in biological samples are required.

A biomarker method was developed and validated on a 4000 QTrap LC-MS/MS (AB Sciex) system for analysis of D3G, DON, deepoxy-DON (DOM-1) and DON-3-glucuronide (DON-GlcA) in urine and faeces of rats. Blank urine and faeces samples for method development were collected from male Sprague Dawley rats. Urine was cleaned-up by solid phase extraction and diluted before injection. Faeces samples were freeze-dried, extracted with methanol/water, diluted and injected. The developed LC-MS/MS method uses negative electrospray ionisation and selective reaction monitoring after prior separation of the analytes by reversed phase chromatography on an 1100 series HPLC system (Agilent Technologies). Validation was performed by determination of the apparent recovery, the signal suppression/enhancement, the recovery of the extraction step, the repeatability as well as the limits of detection and quantification.

For the apparent recovery, values of 56%, 77% and 69% for D3G, DON and DOM-1 were assessed in faeces. In urine, values of 45%, 34%, 24% and 89%, respectively, for D3G, DON, DOM and DON-GlcA were determined. Reduced apparent recoveries were caused by severe matrix effects. Based on the expected concentrations in urine and faeces, we conclude that the validated biomarker method can be successfully used in future ADME-studies of D3G.

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Quantitative determination of zearalenone and its major metabolites in animal plasma using LC-MS/MS and (U)HPLC-HR-MS detection

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A sensitive and specific method for the quantitative determination of zearalenone (ZON) and its major metabolites (alpha-zearalenol (α-ZOL), beta-zearalenol (ß-ZOL), alpha-zearalanol (α-ZAL), beta-zearalanol (ß-ZAL) and zearalanone (ZAN)) in animal plasma using liquid chromatography combined with heated electrospray ionization (h-ESI) tandem mass spectrometry (LC-ESI-MS/MS) and high-resolution mass spectrometry ((U)HPLC-HR-MS) is presented. The sample preparation was simple and fast and consisted of a deproteinization step using acetonitrile. Chromatographic separation of the analytes of interest was achieved on a Hypersil Gold column (50 mm x 2.1 mm i.d., dp: 1.9 µm) using 0.01 % acetic acid in water (A) and acetonitrile (B) as mobile phases. A gradient elution was performed at a flow rate of 300 µL min^{-1} and a column temperature of 45 °C.
To obtain a sensitivity as high as possible, both mass spectrometers were operated in the negative h-ESI mode. For the LC-MS/MS analysis, analytes were detected in the selected reaction monitoring mode (SRM), whereas for the (U)HPLC-HR-MS analysis, the instrument was operated in the MS scanning mode (m/z 80 to 800).
Using the LC-MS/MS instrument, the method was in-house validated for all analytes, according to EU guidelines (2002/657/EC): matrix-matched calibration curves were prepared and good linearity ($r \geq 0.99$) was achieved over the concentration range tested (1 - 200 ng mL^{-1}). Limits of quantification (LOQ) were between 1 and 5 ng mL^{-1} for all compounds. Limits of detection ranged from 0.004 to 0.07 ng mL^{-1}. The results for the within-day and between-day precision and accuracy fell within the ranges specified.
The method has been successfully used for the quantitative determination of ZEA in plasma samples from broiler chickens and pigs. α-ZOL was the only metabolite that could be detected, but the concentrations were below the LOQ.
In order to investigate the presence of glucuronide conjugates of ZON and its metabolites, plasma samples were also analysed using a (U)HPLC-HR-MS instrument. In addition, the applicability of the (U)HPLC-HR-MS technique for quantitative purposes was evaluated. A good correlation ($r^2 = 0.9957$) was obtained between the results obtained with the LC-MS/MS and (U)HPLC-HR-MS instruments.
The results prove the usefulness of the developed method for application in the field of toxicokinetic analysis and for exposure assessment of mycotoxins.

Reference

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Highly efficient extraction and clean-up of zearalenone from edible oils

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Zearalenone (ZEN) is a hyperestrogenic mycotoxin that is produced by a variety of *Fusarium* species. Although ZEN contaminates different cereal grains, it is often found on maize. According to the unpolar character of ZEN, particular high concentrations can be found in the oil fraction after maize processing.

To date, the extraction and clean-up of ZEN from edible oil is challenging. Available analytical methods are either very cost intensive or lack specifity. In 2010, Siegel et al. proposed a highly specific and accurate sample preparation technique based on dynamic covalent hydrazine chemistry (DCHC). Carbonyl compounds as ZEN are selectively bound by hydrazine functionalized polymer particles. After the clean-up process ZEN is quantified by HPLC-FLD.

The aim of this work was to compare different resins for their suitability in the DCHC clean-up process.

Seven different resins were obtained and compared in this study. Five of them were commercially available, whereas two resins were custom synthesized. All resins have different properties regarding their support material, particle size, pore size, specific surface area, loading and swelling. The kinetic experiments performed, involve coupling and decoupling of ZEN over 120 min of time and are based on the protocol described in Siegel et al., 2010.

Two of the seven resins, one based on silica and the other based on polystyrene-divinylbenzene, showed superior kinetic performance and are therefore chosen for further experiments. Since the DCHC approach allows the resin to be reused, it might be suited for an automatic SPE-HPLC online system. In addition to further improvement of the reaction kinetics, the conditions for reconstitution of the SPE material are subject to future investigations.

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Bioassay based on *Gliocladium roseum* zes2-GFP strain responds to zearalenone but not other estrogens

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The soil borne mycoparasite *Gliocladium roseum* (syn. *Clonostachys rosea*) is extensively studied as a biocontrol agent against fungal diseases of plants. Due to the secretion of cell wall degrading enzymes, such as chitinases, and the synthesis of gliotoxin *G. roseum* is an efficient antagonist of filamentous fungi. Furthermore, the mycoparasite is able to detoxify mycotoxin zearalenone (ZEA) by secretion of a specific lactonase. ZEA, which is consistently produced among strains of *Fusarium graminearum, F. culmorum* and *F. crookwellense,* binds to cytoplasmic estrogen receptors in mammals and is known to have carcinogenic as well as genotoxic activities.
Recombinant *G. roseum* strain carrying a fusion of the ZEA-sensing promotor-element (zes2) with a reporter gene (green fluorescent protein (GFP)) was constructed using *Agrobacterium tumefaciens*-mediated transformation. To investigate the suitability of the *G. roseum* zes2-GFP fusion strain for quantitative determination of ZEA concentration in complex matrices, maize plants were grown under field conditions and artificially infected with zearalenone-producing *Fusarium* ssp. Harvested cobs were oven-dried, homogenized and extracted with a mixture of acetonitrile and water (75:25, v/v), followed by clean-up with immunoaffinity columns (ZearaStar, Romer Labs) as well as solid-phase extraction (SPE) columns (Bond Elut Mycotoxin, Agilent). The solvent was evaporated and the residues redissolved in methanol/water (40:60, v/v). Spore suspension of the *zes*2-GFP fusion strain was added to GM7 liquid medium in a 96-well flat bottom microplate. The cultures (54 µl total volume) were incubated under dark conditions at 25°C. After 48 h of incubation, 6 µl of undiluted sample extract (10%) as well as 3 dilutions of the extract were added to the cultures. Fluorescence of GFP was measured with a fluorescence reader (FLX800, Biotek) at 24 h, 36 h and 48 h. To investigate the kinetics of the assay for a broad range of ZEA concentrations, we monitored the fluorescence every 12 h for 144 h. To evaluate the suitablility of the assay for fungal strain screening for ZEA production, we cultivated 38 fungal strains on autoclaved rice kernels for 40 days and extracted the samples with acetonitrile/water (75:25, v/v). The solvent was transferred into microplates and evaporated. GM7 medium containing spore suspension of *zes*2-GFP fusion strain was added to the residues. Fluorescence was measured after 48 h of incubation.

We found an approximately linear relation between ZEA concentration in maize samples and fluorescence signal after clean-up for samples containing up to 4 µg ZEA/ml using immunoaffinity columns. The assay is therefore suitable even for complex matrices. Inexpensive cleanup using SPE is possible, too. In a screening of fungal strains, a clear division into ZEA-producers and non-producers was possible after 48 h of incubation.

Preparation of ergot alkaloid containing mixed feeding stuff and use as reference materials

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Ergot alkaloids are mycotoxins that are produced by all species of the Claviceps genua, most notably by Claviceps purpurea. The fungus infects the grain ears of various gramineous plants such as wheat, rye, triticale, barley, and millet. Instead of sane grains large discoloured sclerotia (secal cornutum) or ergots are formed [EFSA, CFP/EFSA/CONTAM].
The main groups of toxic ergot alkaloids are lysergic acid amids (e.g. ergometrine), ergopeptides (e.g. ergotamine) and iso-ergopeptides (e.g. ergotaminine). The ergot alkaloid pattern and amount vary between fungal strains depending on the host plant, climate and geographical region.
To the present, occurrence data of single ergot alkaloids in feed and food as well as information on toxicological properties are rarely published and still incomplete [EFSA, CFP/EFSA/CONTAM]. In order to generate reliable data, European activities have been launched recently to monitor ergot alkaloids in food and feed [DG SANCO document 10226/2010]. In this regard, an analysis method for the determination of ergot alkaloids in rye and wheat [Müller et al.] was successfully validated in a collaborative trial in 2009. This method is now available in the official compilation of the German Food and Feed Code (Technical rule BVL L15.01/02-5:2012-01). Based on this method a collaborative trial considering feeding stuff analysis is planned in 2012. Previous analyses showed strong interferences with HPLC-FLD resulting in false positive findings caused by the complex composition of mixed feeding stuffs. Thus, the more specific LC-MS/MS will be applied instead. Various mixed feeding stuffs with different levels of ergot alkaloids were prepared in 2011. With the objective to cover a broad range of matrix properties three basic types were selected: 1) cereal based, 2) crude fiber based and 3) fat based materials. The preparation procedure, the challenge "homogeneity" and results will be presented in detail.

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A class targeted metabolomic approach based on liquid chromatography–mass spectrometry for the screening and identification of ergot alkaloids in cereals and cereal products

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Ergot alkaloids are secondary metabolites of fungi of the *Claviceps* genus, most notably *Claviceps purpurea*. Infections are largely prevalent in cereals and wild grasses[1]. After the infection is established, the fungus replaces the developing grain or seed with an alkaloid-containing wintering body called sclerotium or ergot. The alkaloid composition of sclerotia varies largely, depending on the maturity of the sclerotia and on other factors such as the fungal strain, the host plant, the geographical region and the prevailing weather conditions[2]. The sclerotia can be harvested together with the cereal kernels and are thus potentially hazardous to consumers. Commonly used analytical methodologies imply the determination of only a limited number of ergot alkaloids in samples.
In this study a class targeted metabolomic approach based liquid chromatography–mass spectrometry was applied to investigate ergot alkaloids in various cereal and cereal products. Initially, an LTQ ion trap mass spectrometer equipped with an electrospray ion source was used to study the fragmentation pattern of ergot alkaloids in the positive ionization mode. A fragment with *m/z* 223, corresponding to the ergolene ring, was found to be a characteristic ion for ergot alkaloids. Parent scan monitoring of the *m/z* 223 ion using liquid chromatography–tandem mass spectrometry on a triple quadrupole instrument allowed the detection of several ergot alkaloid derivatives. Further identification of each derivative was performed coupling liquid chromatography with orbitrap high resolution mass spectrometry. The reported approach represents a suitable tool for the detection of unknown or untargeted *Claviceps purpurea* metabolites and can be applied for focused metabolomics screening of variant or mutant strains.

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Development of analytical methods for the determination of tenuazonic acid analogues in food commodities

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Introduction: The mycotoxin tenuazonic acid (TA) is a tetramic acid on the basis of isoleucine and is produced by moulds of the genus *Alternaria*, predominantly. TA exhibits several toxicological effects (cytotoxic, phytotoxic, bactericide) and is also acutely toxic to animals. TA recently has been found in different food commodities like cereals, beer, tomato products, fruit juices and spices. Beside TA formation, it has been reported that *Alternaria tenuis* and *Pyricularia oryzae* can also produce the valine (ValTA) and the leucine analogue (LeuTA). At least ValTA has been found as co-occurring contaminant along with TA in tomatoes [1] and rice [2], already. Thus, it was the aim of this study to develop analytical methods to determine TA analogues of different amino acids in food commodities.

Results and Discussion: The TA analogues derived from alanine (AlaTA), valine (ValTA), leucine (LeuTA), phenylalanine (PheTA), glutamic acid (GluTA), aspartic acid (AspTA) and lysine (LysTA) were synthesized as racemates using *Dieckmann* condensation of the respective *N*-acetoacetyl amino acid methyl esters. The compounds were purified by preparative HPLC and characterized by mass spectrometry and nuclear resonance spectroscopy (NMR). The concentration was determined by quantitative NRM (q-NMR).
Two LC-MS/MS-methods were developed for the determination of TA analogues in food commodities using an API 4000 QTrap instrumentation. In the first method the TA analogues were separated as anions on a pH-stable stationary phase at pH 9. Fragmentation of the TA analogues in the mass spectrometer was unspecific due to the loss of the side chain of the tetramic acid nucleus. The mass transitions $[M-H]^-$ → m/z 139 and → m/z 112 were used for the determination of all compounds, therefore. The isobaric compounds TA and LeuTA were chromatographically separated to ensure unequivocal identification. However, neither the chromatographic stability (peak shape, retention time) nor the mass spectrometric sensitivity of this method was satisfactory.
Therefore, the second method was based on the derivatization of TA analogues with 2,4-dinitrophenylhydrazin (DNPH) [3]. The resulting derivatives could be detected with high sensitivity using selective mass transitions in general. However, the isobaric TA and LeuTA were not chromatographically separated and the mass transitions were not selective, but the respective fragment ions showed different intensities. Therefore, it was possible to develop a mathematical model based on the intensity ratios of the different fragment ions to differentiate between TA and LeuTA. Analyses of different food commodities (containing TA) revealed only contamination with the TA analogues ValTA and – seldom – LeuTA.

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Hapten Microarray-based Screening of Mycotoxins in Cereals

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Mycotoxins are secondary metabolites of fungi and several are known to be acute toxic, immunosuppressive, mutagenic, teratogenic and carcinogenic. The most important mycotoxins in cereals and maize are deoxynivalenol (DON), ochratoxin A (OTA), aflatoxins (AFB1), zearalenone (ZEA), fumonisins (FB1) and T-2/HT-2. Therefore, the development of a fast and sensitive quantification method for raw food analysis is necessary. Competitive enzyme-linked immunosorbent assays (ELISA) are a viable alternative to the many HPLC-based procedures. However, they do not allow multianalyte testing, generally. Therefore the automated readout system for flow-through chemiluminescence microarrays, the Munich Chip Reader 3 (MCR 3), was developed. The aim is to develop a biochip for the rapid determination of multiple mycotoxins in cereals. In detail, coupleable mycotoxin derivatives are synthesized and then immobilized on PEGylated glass chips. The immunological determination takes place in a flow chamber and the chemiluminescence readout is performed by a CCD camera placed on the top.

In the first experiments the regenerability of the chip, immobilized with OTA, AFB1, DON and FB1, was tested. Therefore, 50 cycles with blank oat extract were run. The results showed that the signal intensities remained above 88%. To analyse the influence of different matrices on the signal intensities as well as on the regenerability of the biochip, additionally 40 regeneration cycles with oat, wheat and maize extracts and MeOH/phosphate buffer (20/80, v/v) for DON were performed. The results clearly showed that the different types of extracts lead to a significantly decreased signal intensity compared with the methanolic phosphate buffer. However, the biochip was regenerable over about 35 cycles with all extracts. In the next experiment, first calibrations are performed with oat extracts spiked with OTA, AFB1 and FB1. The calibration curves showed IC_{50}-values of 5.3 ± 0.2 µg/kg, 3.3 ± 0.3 µg/kg and 618.0 ± 71.0 µg/kg for OTA, AFB1 and FB1. The linear ranges were 2.4 - 12.0 µg/kg for OTA, 1.7 - 7.8 µg/kg for AFB1 and 150.0 – 2861.0 µg/kg for FB1.

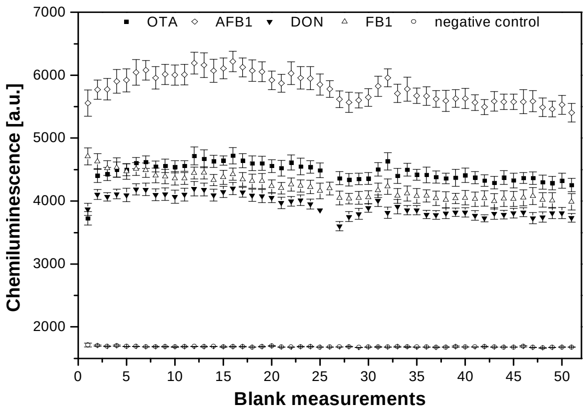

Figure 1: Test of the regenerability of the chip with ochratoxin A, aflatoxin B1, deoxynivalenol and fumonisin B1 in oat extract.

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We thank Dr. R. Dietrich and Prof. E. Märtlbauer (LMU Munich) for providing antibodies against DON and FB1, also for the DON-conjugate.

Identification of genes interfering with alternariol biosynthesis in *A. alternata* by suppression subtractive hybridization

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The filamentous fungus *Alternaria alternata* is ubiquitously present and capable to produce the mycotoxin alternariol (AOH) among others. Typical habitats for *A. alternata* are fruits and vegetables, which usually constitute a high water activity; however cereals with moderate water activity are also frequently colonized by *A. alternata*. The production of secondary metabolites is strictly dependent on environmental factors and characteristics of the habitat. Osmotic components like NaCl show a deep impact on the AOH biosynthesis, resulting in complete inhibition already at low concentrations. High osmolarity in the environment is usually transmitted to the transcriptional level of downstream regulated genes by the HOG pathway including a MAP kinase phosphorylation cascade. It could be demonstrated that the regulation on the HOG phosphorylation level is essential for the inhibition of AOH biosynthesis by changes in the osmolarity. To elucidate the regulatory processes during AOH biosynthesis (low osmolarity) differentially expressed genes were captured by *suppression subtractive hybridization* (SSH). A number of 25 expressed sequence tags (EST) resulted from the AOH biosynthesis condition after comparing to non-productive conditions (high osmolarity). The identified genes were associated with the cell cycle control, mitosis and regulation of the cytoskeleton. Furthermore, the study revealed enzymes for secondary metabolite modification and transporters adequate for cellular export of mycotoxins. At osmotic stress, 6 EST were differentially expressed, mostly associated with stress response. One gene belonged to the *haloacid dehalogenase* (HAD) family, which act antagonistically to kinases by dephosphorylating regulatory enzymes like for example HOG. Further investigations discovered an increasing gene expression of *had* at increasing NaCl concentrations correlated with decreasing HOG phosphorylation and AOH production. Currently, a knock-down of the *had* gene is performed to gain a detailed insight into the function of HAD in the regulation of the AOH biosynthesis.

Simultaneous Determination Of Total Aflatoxin, Ochratoxin A and Zearalenone Using AO ZON PREP® in conjunction with HPLC

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European legislative limits for total aflatoxin, ochratoxin A and zearalenone are in place for cereals and cereal products. There is a demand in the market for faster and less labour intensive tests. Immunoaffinity columns are rapidly becoming the routine standard method of choice for complying with regulatory mycotoxin analysis however there is a growing need for multi-mycotoxin analysis using a single extraction method. In response, R-Biopharm Rhone has produced a multi-toxin immunoaffinity column, AO ZON PREP® enabling the isolation and concentration of aflatoxins B1, B2, G1, G2, ochratoxin A and zearalenone in cereals and cereal products. Two HPLC conditions were developed; one for the simultaneous detection of total aflatoxin and ochratoxin A based on the use of the KOBRA® CELL for derivatisation and a second for zearalenone. The advantage of this new immunoaffinity column is that only one sample preparation method is required for all six mycotoxins therefore having greater sample throughput and a reduction in the use of solvents and consumables.

The method was validated in-house using AO ZON PREP® to test various cereal samples including wheat, maize and rye. Extraction was either with 80% methanol or with 60% acetonitrile, depending on the cereal analysed. Samples were then diluted with PBS before being passed through the immunoaffinity column where they were washed and finally eluted with methanol before being injected onto the HPLC system. Method repeatability was determined and recovery data ranged from 87 - 109% (RSD 2 – 9%). AO ZON PREP® was shown to perform in line with current EU recommendations whilst providing a rapid and robust method of analysis enabling accurate, low level quantification of all six mycotoxins in various cereal samples.

Simultaneous Determination Of Four *Fusarium* Mycotoxins In Beer Using DZT MS-PREP® in conjunction with LC-MS/MS

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Fusarium mycotoxins can be found in a variety of commodities when cereals are stored under adverse conditions including temperature and humidity. Legislation for mycotoxins has recently increased to incorporate additional matrices and toxins, therefore resulting in an increased demand for faster and less labour intensive tests.

Immunoaffinity columns are rapidly becoming the routine standard method of choice for complying with regulatory mycotoxin analysis however there is a growing need for multi-mycotoxin analysis using a single extraction method. In response, R-Biopharm Rhone has produced a multi-toxin immunoaffinity column, DZT MS-PREP®, which enables the isolation and concentration of four commonly occurring *Fusarium* mycotoxins; deoxynivalenol, zearalenone, T-2 and HT-2. The advantages of this new immunoaffinity column are that only one sample preparation method and one single LC-MS/MS run is required for quantifying all four mycotoxins therefore having greater sample throughput and a reduction in the use of solvents and consumables.

The method was validated in-house using DZT MS-PREP® to test various beer samples from Europe. Method repeatability was determined and recovery data ranged from 88 - 105% (RSD 0.8 – 3.9%). DZT MS-PREP® was shown to perform in line with current EU recommendations whilst providing a rapid and robust method of analysis enabling accurate, low level quantification of all 4 mycotoxins in various beer samples from Europe.

Application of a Novel Multi-Mycotoxin Sample Cleanup Method and Stable ^{13}C-Labeled Internal Standards to Improve Accuracy and Sensitivity of Mycotoxin LC-MS/MS Methods

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The need for multi-mycotoxin analyses is constantly rising and the technology of choice is LC-MS/MS. Laboratories are increasingly using LC-MS/MS methods in their routine testing operation. Nevertheless, a problem with LC-MS/MS can be interferences from matrix components leading to differences in analyte ionization. To overcome this ionization effect, internal standards have to be used. The application of fully ^{13}C-labelled internal standards will correct such mass signal intensities between various sample matrices and pure standard calibrants to ensure qualified analysis results. The use of stable ^{13}C-labeled mycotoxins as internal standards has several advantages over another alternative, deuterated (^{2}H) internal standards. Replacing ^{12}C by ^{13}C changes the total mass of the atom only slightly, while using deuterium, the mass doubles, thus, ^{2}H labeled mycotoxins might show retention time shifts, resulting in less accurate LC-MS/MS results.

Nowadays, highly sensitive mycotoxin detection methods are demanded by EU legislation and subsequently by the mycotoxin testing food and feed safety market. For being able to detect multiple mycotoxins at such low detection limits a sample cleanup step should be implemented in the LC-MS/MS method. A novel rapid multi-mycotoxin cleanup method to increase the LC-MS/MS method sensitivity is currently being developed by Romer Labs.

This poster presentation will demonstrate first results of this novel cleanup method development to increase method sensitivity. In addition, it will demonstrate how to use an internal standard for improved accuracy, and furthermore, it will illustrate the importance of applying internal standards when performing quantitative mycotoxin analyses on an LC-MS/MS system.

A Rapid Lateral Flow Quantitative Test for Ochratoxin A in Wheat

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The lateral flow immunochramatographic assay (LFIA) has received much attention in food safety for its rapid screening mycotoxins, veterinary drug residues and even microorganism contamination. To meet market demand, Vicam has developed ochratoxin strip test to rapidly examine ochratoxin A (OTA) in wheat. OTA is a metabolite of fungus such as *Aspergillus ochraceus* and *Penicillium viridicatum*, and is considered as a nephrotoxin and a potent renal carcinogen in rodents. Several countries have set limits for OTA in wheat, cereals and coffee.
The current strip test is a rapid, simple, and quantitative test for OTA in wheat. The grounded wheat sample is simply extracted in 70% methanol; extract is filtered and mixed with diluent (1:1) and then 100µl of diluted solution is applied to the LFIA testing cassette; the result can be read in 5min. Results show that the current ochratoxin strip test has a very high degree of linearity (r^2 = 0.993) with a limit of detection about 2.5ppb, and a testing range of 2.5 to 50ppb. Comparative study indicates that the results generated from current testing procedure correlates very well with the results of HPLC in naturally contaminated wheat. The time required for test is less than 10min including sample preparation.

Multimycotoxin and single Mycotoxin detections using a new solid phase extraction sorbent based on Molecularly Imprinted Polymers

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Clean-up and sample preparation before analysis are key steps in Mycotoxin analysis particularly for its detection at a trace level (range of µg/Kg). Very selective solid phase extraction (SPE) sorbents based on Molecularly Imprinted Polymer (MIP) represents a judicious method that mimics antibodies recognition properties. MIP is a synthetic polymer material able to specifically rebind a target molecule. MIP has the advantages to be not only highly selective and specific but also chemically and thermally stable, compatible with all solvents and cost-effective. This polymer is used as a powerful method for clean-up and pre concentration applications.
The efficiency of a method using MIP as selective sorbents for solid-phase extraction will be shown with respect to the clean-up and pre-concentration in different matrices.
New selective MIP sorbents will be presented, particularly for Fumonisin, Ochratoxin A, Zearalenone and Patulin.
A highlight on Multimycotoxins analysis using Molecularly imprinted polymers will also be displayed for the same matrix.

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Impact of aggressiveness of *Fusarium graminearum* and *F. culmorum* isolates on yield parameters and mycotoxin production

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Fusarium head blight is a serious problem for worldwide cereal production. The main causal agents *F. graminearum* and *F. culmorum* cause yield and quality losses and contamination of grain with the mycotoxins deoxynivalenol (DON), nivalenol (NIV), and zearalenone (ZEA).
Plant-associated isolates from *F. graminearum* and *F. culmorum* of different aggressiveness were inoculated on wheat in field experiments in 2007 and 2008 to ascertain their influence on fungal colonization of the ears, as well as mycotoxin contamination (DON; ZEA; NIV) and yield parameters in the mature crop after inoculation at anthesis with or without irrigation. The isolates were assigned to four different groups of aggressiveness on the basis of pathogenic symptom development and mycotoxin production *in vitro*. Increased levels of Trichothecene-producing *Fusarium* DNA in the ears indicated a successful inoculation of the plants, which resulted in increased DON content in the wheat kernels in 2007. Dry conditions at anthesis markedly suppressed fungal colonization as well as mycotoxin accumulation. However, due to precipitation during the ripening period, yield and thousand-kernel weight were similar whether or not irrigation was applied at the time of inoculation. The level of aggressiveness among the isolates as determined *in vitro* was not reflected in the experiment. In 2008, the *Fusarium* inoculation of wheat heads did not cause fungal growth and mycotoxin contamination in the grain, because of dry weather conditions that occurred over the entire period of anthesis and ripening (Korn et al. 2011).

Additionally, the activity of sucrose-cleaving enzymes, key enzymes in plant defence responses, were measured to check the influences of pathogen attack and irrigation on the plant´s carbohydrate metabolism. The extracellular invertase was found to be the sucrose-cleaving enzyme with the highest activity. The activity of this enzyme was generally increased by irrigation and seemed to augment with rising fungal aggressiveness, but only when the plants were irrigated.

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Fusarium toxins in wheat grains and products from organic and conventional farming

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Due to a high nutritional value of grain, wheat is the most often grown and most common cereal all over the world. Its popularity results from a wide spectrum of cultivars, which makes cultivation possible not only in various climatic zones, but also enables its diverse applications. The wheat grain is used primarily in flour production, yet also in groats or flakes and even as a feed. Such great popularity of wheat makes its protection against diseases and pathogens very important. *Fusarium* fungi being able to produce secondary metabolites (mycotoxins) are the most common cereal pathogens. The majority of mycotoxins are toxic, have adverse influence of human and animals health.

The aim of the present study was to determine the contents of trichothecenes and zealarenone in wheat grain and products (flour, pasta, flakes, bran) originating from organic and conventional crop systems. The study material consisted of 52 organic (27 grain and 25 products) as well as 28 conventional samples (11 grain and 17 products). The samples were collected from Kuyavian-Pomeranian province since September 2009 till April 2011. Mycotoxins (nivalenol – NIV, deoxynivalenol – DON, 3-acetyl-deoxynivalenol – 3ADON, monoacetoxyscirpenol – MAS, diacetoxyscirpenol – DAS, T-2, HT-2, as well as zearalenone – ZEA) were determined using HPLC-MS/MS method. The samples clean-up was performed using Bond Elut® Mycotoxin (Varian) columns and internal standards (IS) ^{13}C-DON, ^{13}C-T-2, ^{13}C-HT-2, and ZAN. Each sample was assayed in triplicates. Among the determined mycotoxins, DON dominated both in raw materials with the highest concentration of 270 ppb – organic and 82.5 ppb – conventional, respectively; as well as products 40.3 ppb – organic and 819 ppb – conventional, respectively. DAS was absent in all samples. ZEA was the most often detected mycotoxin (17 samples of organic grain, 16 samples of organic products, 6 samples of conventional grain, 15 samples of conventional products) with its maximum concentration found in conventional pasta sample at the amount of 21.3 ppb. NIV was the most frequently determined in organic grain (at the maximum level of 27.0 ppb), yet it was also present in organic and conventional products – pasta and bran (one infected sample each – 24.2 ppb and 25.8 ppb, respectively). The T-2 and HT-2 toxins were detected in raw materials and products; however, their highest levels were recorded in organic grain: T-2 – 5.27 ppb, HT-2 – 10.5 ppb and conventional products: T-2 – 3.08 ppb, HT-2 – 20.5 ppb, respectively. MAS was found in organic grain and conventional wheat bran at the maximum concentration of 3.03 ppb and 4.75 ppb, respectively. Bran appeared to be the most infected sample as it contained seven of eight determined mycotoxins: NIV (25.8 ppb), DON (819 ppb), MAS (4.75 ppb), T-2 (2.03 ppb), HT-2 (20.5 ppb), ZEA (3.52 ppb), as well as 3-ADON (<15 ppb). It was concluded that raw materials and products originating from conventional and organic crop systems contained the majority of *Fusarium* metabolites.

Financial support: Ministry of Science and Higher Education project no NN 605 122136

Mycological evaluation of cereals and cereal products from organic farms in 2010

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Progress of organic farming and the increase of demand for high-quality food products has been recently observed. The main aim of organic farming is production of high-quality food that would be pro-health along with reduction of or even giving up the artificial fertilizers and fungicides in agriculture. Nevertheless, production using natural methods, with no plant protection means, arises the risk of infection of the raw material with moulds; and that fact has been confirmed many times in previous studies and literature references.

In 2010, mycological analysis included 126 samples of the following grains: rye (36), oat (27), spelt (17), wheat (15), barley (12), mixture (12), triticale (7), as well as 50 samples of organic products: pasta (15), flour (12), groats (11), flakes (8), and bran (4). All raw material and product samples were collected from Kuyavian-Pomeranian province.

The study aimed at evaluating the level of moulds contamination. Qualitative and quantitative mycological analysis was carried out according to PN ISO 7954: 1999. The highest level of moulds infection was found in barley, while the lowest in wheat samples. Mean number of fungi amounted to: rye – 1.7×10^5 CFU/g, oat – 1.8×10^5 CFU/g, spelt – 3.9×10^4 CFU/g, wheat – 1.6×10^4 CFU/g, barley – 3.0×10^5 CFU/g, cereal mixture – 6.8×10^4 CFU/g, and triticale – 1.7×10^4 CFU/g. Moulds of the following genera were the most numerous in the analyzed samples: *Aureobasidium sp.*, *Penicillium sp.*, *Eurotium sp.* and *Cladosporium sp.* Barley samples were dominated by moulds of *Penicillium sp.* - 23% and *Eurotium sp.*- 19%, wheat - *Penicillium sp.* - 24% and *Aureobasidium sp.* - 18%, rye - *Penicillium sp.* - 34% and *Aureobasidium* - 33%, cereal mixture - *Cladosporium sp.* – 22%, *Aureobasidium sp.* - 17%, and *Eurotium sp.*– 16%, triticale - *Aureobasidium sp.* – 26%, and *Eurotium sp.* 21%, spelt - *Aureobasidium sp.* and *Alternaria sp.*, as well as oat - *Cladosporium sp.*– 36% and *Penicillium sp.* – 19%. Quite high levels of moulds and yeast (2.1×10^5 CFU/g, on average) were recorded in the studied samples with the highest in rye and barley flour and multi-cereal flakes. All groups of products were dominated by moulds of *Acremonium sp., Mucor sp., Syncephalastrum sp.*, and *Penicillium sp.* genera. The scope of mould infection in pasta samples amounted to <100, K – 1.8×10^6 CFU/g, in flour <50, K – 9.3×10^5 CFU/g, in groats 1.5×10^2 – 4.6×10^5 CFU/g, in flakes 1.8×10^3 – 1.3×10^6 CFU/g, and in bran 1.2×10^2 – 1.7×10^5 CFU/g.

The field type of *Fusarium* occurred very rarely; their highest percentage was found in cereal mixture – 9% and barley – 5%. *F. poae, F. equiseti, F. tricinctum, F. culmorum, F. oxysporum, F. sporotrichioides, F. semitectum, F. verticillioides, F. proliferatum* were the most often isolated in the cereal samples. Of all organic products only pasta and flour samples were contaminated with *Fusarium,* yet the level of these fungi constituted only 2% of the total number of moulds.

However, it was concluded that both organic raw materials and products were highly infected by storage (*Penicillium sp., Eurotium sp.*) and field moulds (*Aureobasidium sp., Cladosporium sp.*).

Financial support: Ministry of Science and Higher Education project no NN 305 122136

Fusarium toxins in bread cereals of the federal state Brandenburg harvested in the years 2000-2011. Comparison of integrated and ecological cultivation

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Introduction: To record the quality of bread cereals of integrated and ecological cultivation of the federal state of Brandenburg over the period of twelve years (2000-2011), both wheat and rye samples were analysed for *Fusarium* toxins.

Materials and Methods: During the twelve years, appr. 60 wheat and 60 rye samples, collected from farmers of all the agricultural districts of the federal state with special consideration of ecological cultivation, were analysed. The *Fusarium* toxins deoxynivalenol and zearalenone were analyzed using HPLC methods. Since 2009 further mycotoxins were analyzed by means of LC-MS/MS (nivalenol, T-2 and HT-2 toxins). Since 2010 the DON derivatives 3-Ac-DON and 15-Ac-DON were additionally determined and all analyses were done by LC-MS/MS.
Since 2007 investigations were made to estimate in advance the mycotoxin status of the harvest. These estimations were made by monitoring appr. 50-70 pre-harvest samples (winter wheat and triticale) from fields with high Fusarium risk.

Results and discussion: DON was detected in wheat for all years of the investigation. Over the years the measured contents of DON varied widely. Zearalenone and nivalenol were rarely detected. T-2 and HT-2 toxins, 3- Ac-DON and 15-Ac-DON were detected only occasionally and in very low concentrations.
2002 and 2007 were years with very high DON contaminations. In the other years the DON contamination was significantly lower.
The DON contamination of the pre-harvested samples from risk fields indicated the situations which were to be expected in the samples from normal fields. In years with high contamination the samples from risk fields contained very high DON levels and in years with low contamination the samples from risk fields also had lower levels.
The content of mycotoxins in rye was significantly lower than in wheat.
Over the twelve years, differences between samples of ecological cultivation and integrated cultivation with regard to the frequency and the measured contents were observed. The contamination by *Fusarium* toxins was significantly less frequent and lower in cereals of ecological cultivation than in cereals of integrated cultivation.

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Occurrence of *Fusarium* species and mycotoxins on spelt grain depending on cultivar and cultivation

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Spelt (*Triticum spelta* L.) is one of ancient wheats cultivated in Europe and North America mainly due to the nutritional value. High quality grain-based products are very important element of well-balanced, healthy diet and main food products throughout the world (Botallico, Perrone 2002, Castoria at al. 2005). Spelt is infected by fungi from genus *Fusarium*, producers of mycotoxins. The spelt is more resistant to infection by the fungi than wheat. Spelt resistance to fungal infections may be related to the polyphenols content in grain. These compounds are involved in the production of cell wall lignification which becomes a physical barrier to fungi infection (Kohajdová and Karovicová 2008).
The aim of this study was identification of *Fusarium* species in the grain of two spelt cultivars with different cultivation and the determination of mycotoxins content in this grain.
In the study two cultivars of spelt with different susceptibility to *Fusarium* head blight were used i.e Franckenkorn and Obelkulmer Rotkorn. The cultivars were cultivated with undersown clover and without the plant. Forecrop was spring wheat.
Fusarium species isolated from spelt grain were identified using conventional mycological analysis. The ELISA method was used to determination in infected grain following mycotoxins: deoxynivalenol, T-2 toxin and zearalenone.
The most number of fungi from genus *Fusarium* was isolated from spelt grain cv Oberkulmer Rotkorn cultivated with undersown clover. From spelt grain cv Franckernkorn cultivated with undersown clover only not sporulated mycelium *Fusarium* spp. were isolated.
Conventional mycological analysis of spelt grain showed that main cause of *Fusarium* head blight was *Fusarium poae*. Accompanying fungi in disease causing were *F. nivale, F. avenaceum* and *F. graminearum*. Cultivar and way of cultivation had significant impact on the fungi occurrence on grain.
Franckerkorn was less susceptible to mycotoxin contamination than Oberkulmer Rotkorn. The highest content of zearalenone was found in Oberkulmer Rotkorn cultivated without undersown clover. Franckerkorn cultivated with and without undersown clover and Oberkulmer Rotkorn with undersown clover were not contaminated by ZEN.
Content of deoxynivalenol, zearalenone and T-2 toxin depended on cereal cultivar and way of cultivation. In spelt with undersown clover increase of T-2 toxin and DON and reduction of ZEN concentrations in grain of cv. Oberkulmer Rotkorn were observed. Mycotoxins contamination of spelt grain from object without undersown clover correlated with *Fusarium* spp. occurrence on grain. More susceptible to infection by *Fusarium* fungi cv. Oberkulmer Rotkorn characterized by higher mycotoxins content in grain than less susceptible to the fungi infection cv. Franckerkorn.

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Performance of German winter wheat cultivars in mycotoxin reduction and resistance against *Fusarium* head blight

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Fusarium head blight (FHB) caused by *Fusarium graminearum* (Schwabe) and *Fusarium culmorum* (W.G. Smith) belongs to the most damaging diseases in cereal crops. Actually mycotoxin contamination of human food and animal feed became a more important feature than direct yield losses that often occur irregularly. *F. culmorum* was described to produce deoxynivalenol (DON), but also the ability to produce DON derivatives and zearalenone has been recognized in some isolates of both species (Mirocha et al., 1994; Sýkorová et al., 2003). Different studies revealed significant relations between DON content and characteristics of FHB infection (Miedaner et al., 2001). However the relationship between disease symptoms and DON content is not yet well understood in every case. Using molecular technologies with the possibility to determinate the amount of pathogen DNA could be a tool for better identification of resistance levels and to understand factors that influence FHB. This could be important for minimizing the risk of mycotoxin contamination of grains and foodstuffs.
For official cultivar disease ranking, the susceptibility of German cultivars and new breeding lines against FHB was evaluated every year. Under field conditions the FHB nursery was investigated at six different sites in Germany.
Furthermore, the FHB and AUDPC were calculated using both incidence and severity. In some cases the DON contamination of the grains was also analyzed.
New registered winter wheat cultivars in 2010 and 2011 showed FHB symptoms between 5 to 55% assessed 28 days after inoculation. Analysing the harvested grains revealed DON contents up to 7.4 mg/kg. A strong correlation coefficient (r=0.74**) was observed between the visual disease symptoms and the mycotoxin content. In accordance to this data a ranking for FHB and DON on a scale from 1-9 (1= low susceptible / low toxin; 9= high susceptible / high toxin) could be made. Among the cultivars evaluated in this study 'Toras', 'Impression' and 'Opal' were the most resistant cultivars, whereas 'Ritmo', 'Tobak' and 'Xantippe' were the most susceptible with high DON contamination. To conclude, results of this study suggest that using less susceptible cultivars to FHB reducing mycotoxine (e.g. DON) concentration by 50-70%. The performance of resistance cultivars in reduction of deoxynivalenol is comparable to the efficacy of fungicides used for fusarium head blight control.

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Mycotoxicological evaluation of rye and its products

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Rye (*Secale cereale* L.) can be considered as a typically European cereal with up to 90% of crops originating from a region stretching from North-German Lowlands to the Ural and about 20% of a worldwide production in Poland.
Unfortunately, rye utilization as feed for animals has been recently dramatically reduced. It may result from the fact that the grain contains the so-called *anti-nutritional factors*, compounds able to disturb a proper digestion and metabolism processes. However, it has not been confirmed that they have negative impact on human health. Consumption becomes dangerous when rye grain is infected with such diseases as ergot and head blight. In the case of the latter, mycotoxins of moulds from *Fusarium* genera, are the most serious threat.
The study aimed at analyzing the mycotoxins content in rye grains and its products originating from organic and conventional crop systems in 2009-2011. The material consisted of 94 samples, including 19 from conventional farms (7 – raw material and 12 - products), whereas 75 from organic crop systems (52 – raw materials and 23 - products). The food products (flour, bran, flakes, pasta) were purchased from local shops in Kuyavian-Pomeranian province. Most of raw materials also came from the same region. Each sample was assayed in 3 replicates. Eight mycotoxins were determined using HPLC-MS/MS method: nivalenol (NIV), deoxynivalenol (DON), 3-acetyldeoxynivalenol (3ADON), monoacetoxyscirpenol (MAS), diacetoxyscirpenol (DAS), T-2 and HT-2 toxins and zearalenone (ZEA). Sample preparation was made using Bond Elut® Mycotoxin columns (Varian) and internal standards (IS) ^{13}C-DON, ^{13}C-T-2, ^{13}C-HT-2, ZAN.
Among 8 determined mycotoxins, two were of major importance: DON and ZEA. DON was present at its highest concentration in the analyzed samples; its maximum level of 140 ppb was found in organic raw material. In that group, the percentage of the infected samples was 37%. Of all food products, the conventional ones appeared to be more contaminated (percentage of infected samples amounted to 75%) with the maximum level of 97.0 ppb in rye bran. The largest number of samples was contaminated with ZEA, detected in up to 83% of conventional raw material samples, with the highest concentration of 1.96 ppb and in 46% of organic raw material samples – at the highest level of 72.4 ppb. No analyzed sample contained 3ADON or DAS, while NIV and MAS concentrations were close to the detection limit. Higher contents of T-2 and HT-2 toxins were found only in organic raw material at the maximum levels of 24.8 ppb for T-2 and 24.3 ppb for HT-2. Both raw materials and products were contaminated at similar degree. For instance, mean concentration of DON in conventional products (25.3 ppb) was slightly higher than the corresponding value in raw material (<15 ppb). In the case of ZEA, its level in conventional products (0.70 ppb) was slightly lower as compared to the raw material (0.82 ppb). In organic products and raw materials, the average DON concentration was low and below <15 ppb, however mean ZEA content was three times as high as in raw materials (2.1 ppb) compared to products (<0.6 ppb). No tested sample exceeded the maximum acceptable levels of mycotoxins (Commission Regulation 1881/2006 EC).
Financial support: Ministry of Science and Higher Education Project No. NN 305 122136

Variation of T-2/HT-2-toxin levels in artificially infected barley during the growing season

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Introduction: Recent surveys in the northern European countries have indicated that the levels of T-2 and HT-2 in cereal samples have increased during the past few years (Edwards *et al*., van der Fels-Klerx). Recognising the emerging risk, the European Commission is currently discussing the regulatory limits for the maximum levels of these acutely highly toxic compounds. *Fusarium sporotrichioides* and *F. langsethiae* are considered as the principal producers of T-2 and HT-2 in small grain cereals, and in particular, *F. langsethiae* is becoming more widespread in Scandinavia as well as in other parts of Europe (Edwards et al., van der Fels-Klerx). In this study the variation T-2 and HT-2-toxins during the growing season was studied from artificially infected barley samples.

Materials and methods: Three varieties of barley (Saana, Scarlett, Tipple) were inoculated with *F. sporotrichioides* at anthesis. Samples were collected at several time-points during the growing seasons of 2010 and 2011, and analysed for HT-2/T-2 by using LC-MS/MS.

The ground barley samples were extracted with 84 % acetonitrile in water solution and the extracts were filtered. An aliquot of 5 ml was withdrawn and evaporated to dryness using nitrogen flow. The samples were redissolved in methanol/water-solution and the filtered samples were analysed by LC-MS/MS.

Results and conclusion: In 2010 the highest amount of T-2 and HT-2-toxin in the inoculated samples was 680 ppb and 400 ppb, respectively. In the control samples the concentrations were 150 ppb and 65 ppb. T-2 and HT-2 were produced rapidly, as they were detectable already at 11 days after inoculation. The concentration of HT-2-toxin was increasing throughout the growing season, whereas the concentration curve for T-2 followed the quadratic form in all varieties. The level of T-2 was higher than HT-2 in Saana and Scarlett at first but later during the growing season the production rate of T-2 was decreasing. An inverse ratio of T-2/HT-2-toxin in Tipple was observed. It has been hypothesised that fungus itself could degrade T-2 to HT-2 affecting the ratio of these two (Medina *et al*.). The preliminary results for the year 2011 show a similar trend in T-2/HT-2-toxin concentrations.

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Statistical aspects of the occurrence, distribution and correlation of ergot alkaloids and sclerotia in German rye from harvest 2010 and 2011

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Every year the Max Rubner-Institut (MRI) -Federal Research Institute of Nutrition and Food- analyses statistically taken samples from 12 (13) different Federal States in Germany în the framework of the specific harvest and quality assessment (BEE). Samples were taken during harvest in the field and directly sent to MRI.
Three rye samples were pooled after grinding (500µm) and screened for ergot alkaloids with the LC-MS/MS-method[a] with a detection limit of 1µg/kg. Positively screened pools were separated and further analysed individually[b].

Table 1 gives an overview about the number of rye samples, the analysed samples and the range of ergot alkaloid content and sclerotia content of the harvest 2010 and 2011. There were fewer samples positively tested for ergot alkaloid in 2011, but the contents of ergot alkaloid and sclerotia were higher compared to 2010.

Tab.1: Overview about the number of rye samples and the range of ergot alkaloid and sclerotia content

		Harvest 2010	Harvest 2011
Number of rye samples		238	229
Number of positively tested rye samples		179	112
Amount		75%	49%
Ergot alkaloid content	min.	0 µg/kg	0 µg/kg
	max	2539 µg/kg	6318 µg/kg
Sclerotia content	min.	0%	0,01%
	max	0,45%	1,76%

The statistical analysis shows significant differences between the regions in Germany for ergometrin, ergotamine/in and ergocristin/in in the harvest 2010, but any significant differences in samples from harvest 2011.
There are correlations between the ergot alkaloid content and the contents of ergosine, ergotamine/ine, ergocornine/ine, and ergocryptine/ine in 2010, but any correlations in 2011. This shows that the occurrence and distribution of ergot alkaloids varies from year to year and depends on individual growing conditions. We didn't find any correlations between ergot alkaloid content and the sclerotia amount in both years of harvesting.

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Occurrence of *Fusarium* species and mycotoxins in wheat and maize in Schleswig-Holstein 2011

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Maize and wheat are frequently attacked by *Fusarium* species, causing ear and stem root in maize and *Fusarium* head blight (FHB) in wheat, respectively.
In the year 2011 grains of wheat (variety "Ritmo") and maize silage (whole plant, varieties "Lorado", "LG 30222", "P 8000", "Torres") from locations all over Schleswig-Holstein were analyzed for the occurrence of different *Fusarium* species and for their mycotoxin content. This results were combined with the potential influence of different cropping factors (variety, crop-rotation and cultivation), as well as climatic influences. Samples were obtained from a monitoring program in cooperation with the *Landwirtschaftskammer* Schleswig-Holstein. Ten locations for maize and seven locations for wheat cultivation were used. Mycotoxins (A- and B-trichothecenes, zearalenon) were determined by LC/MS.
In wheat five *Fusarium* species and were detected at all locations: *F. graminearum*, *F. culmorum*, *F. poae*, *F. crookwellense* and *F. langsethiae*. *F. equiseti* was detected at only one location. The mean content of deoxynivalenol (DON) was 2126 µg/kg (range: 914 to 3182 µg/kg) and samples from six of seven locations were above the European limit for unprocessed grains (1250 µg/kg). The contamination of zearalenon (ZEA) was 518 µg/kg (range: 392 to 657 µg/kg) and exceeded the European limit for unprocessed grains about five times (100 µg/kg). Nivalenol (NIV) was detected at six locations (mean: 100 µg/kg, range: 0 to 167 µg/kg). Other mycotoxins (e.g. T2, HT2, fusarenon X) were not detected.
In maize silage the *Fusarium* species *F. graminearum*, *F. culmorum* and *F. poae* were ubiquituosly present followed by *F. avenaceum*, *F. crookwellense*, *F. langsethiae*, *F. sporotrichioides* and *F. equiseti* which were not present at all locations. Similar to wheat only the three mycotoxins DON, NIV and ZEA were detected. The average content of DON for all ten locations for variety "Lorado" was 6765 µg/kg DM (range: 1225 to 26068 µg/kg DM), for ZEA 1791 µg/kg DM (range: 671 to 5991 µg/kg DM) and for NIV 990 µg/kg DM (range: 183 to 2720 µg/kg DM). The effect of cropping systems showed that the combination of pre-crop maize and conservation tillage results in the highest mycotoxin concentrations in comparison to crop-rotation and plowing. The cultivation of a more tolerant variety ("LG 30222", "P 8000", "Torres")) reduces the content of DON, NIV and ZEA. But at locations where conservation tillage and pre-crop maize was used, the content of DON, NIV and ZEA was similar.
The results demonstrated that the cultivation of low susceptible maize varieties, crop-rotations and plowing will be a tool to reduce mycotoxin contaminations.

Fungicide effects against *Fusarium* spp. in maize 2011

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In recent years, the economic importance of maize as a forage and energy plant has increased accompanied by an extension of the maize cultivation in Germany.
Although the culture is commonly known as healthy, its high occurrence results in a significant disease pressure caused by several pathogens to other cultures (Deutsches Maiskomitee, 2011). One of the most important diseases in maize is *Fusarium* spp.. Among others, it mainly produces mycotoxins belonging to the group of trichothecenes as well as zearalenon. Thus it causes negative impact of the food chain.
Aim of this project is to examine the effect of fungicides against these pathogens on yield and quality, although in Germany no fungicides against maize diseases are currently registrated. In order to investigate the climate-influence on the infestation of *Fusarium* spp. in maize, field trials were set up at two locations. The first site was in Schleswig-Holstein with a maritime climate. The other site was in Bavaria with a more continental climate as in Schleswig-Holstein. For both trials the cultivar "Lorado" was used.
The plots were treated with an azole in combination with a strobilurine at the growth stages GS 34/37, GS 51/55 and GS 34/37,51/55,65. The harvested material CCM (corn-cob-mix) was analyzed for its content of deoxynivalenol (DON) and zearalenon (ZEA) by HP/LC and LC/MS.
The distribution of *Fusarium* spp. was benefit by sufficient humidity and temperatures between 11-30°C in 2011 (Leonard & Bushnell, 2003). Regarding this fact in combination with conservation tillage a strong impact of mycotoxins has been found in maize in Schleswig-Holstein. In Bavaria minor humidity occurred and plow was used, so the maize plants did not contain as high levels of mycotoxins as found in Schleswig-Holstein. In 2006 the European Commission determined orientation values for mycotoxin contents in feeding stuff. For piglets and gilts the value is set to on 900 µg/kg DM for DON and 100 µg/kg DM for ZEA. The results of mycotoxin analysis from 2011 for CCM clearly exceed these values. In the untreated control 11755,36 µg/kg DM DON and 28608,8 µg/kg DM Zea could be measured. A reduction ≤ 91 % of DON/ZEA could be detected by applying the most efficiently treatment during ear emergence to flowering (GS 55).
Additionally an abundance of different *Fusarium*-species could be detected by PCR using specific primers. These results clearly indicate that a permission for fungicides in maize is necessary.

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Survey of fungal metabolites in 90 maize samples from four agro-ecological zones in Malawi determined by LC-MS/MS

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Maize is a major staple food in the in the Sub-Saharan region of Africa. In Malawi, maize contributes significantly to diets of more than 80% of the population, with per capita consumption of about 180 kg per year (500g/person/day). With such consumption trends dietary exposures to mycotoxins occurring in maize and associated health risks are more likely to be high considering that Malawi is situated in the tropics where adverse climate favors fungal and mycotoxin contamination. Unfortunately, few surveys have been conducted in Malawi to determine the prevalence of fungal and bacterial metabolites in food products and all the recorded studies only focused on aflatoxins. As Malawi comprises a narrow and long stretch with four distinct agro-ecologies, different patterns of fungal metabolites are to be expected in these regions.

The objective of this study was to investigate the natural occurrence of a broad spectrum of mycotoxins and other fungal metabolites in maize from four different agro-ecologies of Malawi by applying a multi-mycotoxin method based on liquid chromatography-tandem mass spectrometry. Samples were collected from farmsteads of 15-20 villages per district in high altitude districts (Mzimba and Dedza), mid altitude (Lilongwe) Lower altitude (Nsanje) and lake shore (Mangochi).

As expected, aflatoxins were observed to be the biggest threat with aflatoxin B1 ranging up to 600 µg/kg. However, there was a significant difference between the districts with hot climate (90 and 70% of positive samples, respectively) and the other three districts (0-15% positive samples). Fumonisins exhibited high prevalence in samples from all districts, but only few samples had concentrations exceeding the limits set in the EU. Similar to the aflatoxins, a statistically significant difference was observed between the samples collected from districts with a hot and a moderate climate, respectively. Zearalenone exhibited a reversed trend, with a considerably fraction of the samples from three districts exceeding 200 µg/kg. All the other mycotoxins addressed by regulations were far below the related limits with very few exceptions (e.g. a sample from Mangochi exhibited 15.8 µg/kg ochratoxin A).

As considers the overall metabolite spectrum, a total of 76 metabolites were identified in the samples, including emerging *Fusarium* toxins, metabolites from the aflatoxin biosynthesis pathway and other metabolites from *Aspergillus flavus* and *Aspergillus parasiticus* (3-nitropropionic acid, cyclopiazonic acid, kojic acid, aflatrem) as well as *Penicillium* metabolites. The most interesting findings were the large concentrations of nivalenol in comparison to deoxynivalenol in some samples, large concentrations of citrinin in samples from hot districts (882±304 µg/kg in samples from Mangochi) and the prevalence of rugulosin and skyrin (these compounds are considered as toxins produced by *Penicillium islandicum*).

The determination of nine trichothecenes and zearalenone in foods in the Czech Republic in the years 2009-2010

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Introduction:
A study „SAFEMON" of the occurrence of trichothecenes A and B and zearalenone in selected foods was prepared. Investigations in this study which is a part of Total Diet study were incorporated into the National Environment and Health Monitoring Programme in the Czech Republic. Its objective was to obtain information on the degree of the current contamination of foods with trichothecenes A and B and zearalenone (ZEA).

Materials and Methods:
Thirty-four commodities (e.g. cereal products, flour, oat flakes, whole bread, bread, rolls, muesli, biscuits, pastry, beer) were collected at 12 places in four time periods in Czech retail in the years 2009-2010. It represents a total of 480 food samples.

The modified, validated and accredited (CSN EN ISO/IEC 17025) LC-MS/MS method was employed for purposes of the determination of nine trichothecenes A and B (DON, 3-AcDON, 15-AcDON, DON-3-Glu, NIV, T-2 toxin, HT-2 toxin, DAS, FUS-X) and ZEA (Berthiller *et al.* 2005; Biselli *et al.* 2005). The food sample was extracted with acetonitrile/water (84:16, v/v). Swift clean-up of food samples was performed with MycoSep 226 columns. The limits of quantification (LOQ) of the methods were between 0.05-1.7 ng/g.

Results:
Of the 480 analyzed samples, the incidence of DON, DON-3-Glu, FUS X, NIV, T-2 and HT-2 toxin was 85, 73, 9.7, 5, 2.7 and 2.7 %, respectively; levels detected ranged from 0.44–331, 0.47–186, 1.40–34.1, 0.77–7.0, 0.69–12.8 and 1.01–3.5 ng/g, respectively. The results of 3-AcDON, 15-AcDON, DAS and ZEA were under the limit of quantification (LOQ) of the method.

Conclusions:
Mycotoxins contents that were detected were similar to other research studies in Europe.

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This study was supported by the National Institute of Public Health, Center for Health, Nutrition and Food in Brno.

Producers and important dietary sources of ochratoxin A – a recent information

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Introduction:
Ochratoxin A (OTA) is a very important mycotoxin and its research is oriented right now on the new finding of OTA like complete carcinogen, information about OTA producers and new exposure sources of OTA.

OTA producers:
A very large number of microfungi species have been claimed to produce OTA and are in need of revision. The results of the revision are following: OTA is produced in foodstuffs by *Aspergillus* Section *Circumdati* (*Aspergillus ochraceus, A. westerdijkiae, A. steynii*) and Aspergillus Section Nigri (*Aspergillus carbonarius, A. foetidus, A. lacticoffeatus, A. niger, A. sclerotioniger, A. tubingensis*) above all in subtropical and tropical areas. OTA is produced in foodstuffs by *Penicillium verrucosum* and *P. nordicum* above all in temperate and colder zones.

Important dietary sources of OTA:
OTA was frequently detected in both foodstuffs of vegetable origin (e.g. cereal products, beer, coffee, cacao, dried red pepper, chilli powder, black pepper, cayenne pepper, nutmeg, coriander, ginger, curcuma, legumes, liquorices, green tea, figs, raisins, grape juice, wine), and in foodstuffs of animal origin (e.g. pork meat, pork blood products, pork kidney, pork liver).

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This study was financially supported by the project (no. NT12051-3/2011) from the Czech Ministry of Health (IGA MZ CR)

Dietary exposure of ochratoxin A for ten population groups in the Czech Republic – a new research project

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Introduction:
According to recent knowledge about ochratoxin A (OTA) carcinogenicity, information about new OTA exposure sources and recent results of urinary OTA excretion in men and women is realised new research project: *„Ochratoxin A – health risk assessment for selected population groups in the Czech Republic"*. The project objective is an assessment of dietary exposure and health risk characterization of OTA for ten population groups of age 4-90 years, both sexes.

Methods for data collection, analysis and statistical tools:
There is planned sampling of 76 types of foodstuffs - significant exposure sources of OTA (e.g. cereals, spices, coffee, cacao, beer, wine, raisins, pulses, meat, meat products, edible offal) in the project proposal. It represents 912 foodstuffs samples. Sampling will be undertaken according to schedule in 4 sampling terms in the years 2011-2012 at 4 regions of the Czech Republic. The samples will be taken off randomly, not-proportionally to the whole batch, in retail such a way like does it a common consumer. 80% of foodstuffs will be purchased in town agglomeration and 20 % in rural community.

Validated and accredited ultra-trace methods for determination of OTA in foodstuffs – significant exposure sources of OTA will be developed. OTA in foodstuffs will be determined by high performance liquid chromatography method with fluorescence detection (HPLC-FD). As a sample clean-up procedure immunoaffinity chromatography (OCHRAPREP®, R-Biopharm) will be used. Limit of quantification of the method (LOQ) will be varied between ones and hundreds ng/kg according to a type of sample matrix. Quality of laboratory results will be provided by using certified reference materials and by participation in international inter-laboratory proficiency testing (FAPAS).

For the determination of food consumption will be used data from the study of individual food consumption (SISP04) that gives values of average intake of individual foodstuffs for single groups of population in the Czech Republic. It is concerned with ten population groups of age 4-90 years, both sexes.

For the point assessment of dietary exposure in ng/kg.b.w./day - week will be used analytical data of OTA in foodstuffs at the level of median, arithmetic mean and 90% percentile and values of the average intake of individual foodstuffs for single groups of population in the Czech Republic.

The obtained results will serve to expansion of the knowledge level in the assessment of dietary exposure and characterization of OTA risk for population groups in the Czech Republic on the base of the newest knowledge about OTA-DNA adducts formation and OTA carcinogenicity.

This study was financially supported by the project (no. NT12051-3/2011) from the Czech Ministry of Health (IGA MZ CR)

Deoxynivalenol and zearalenon in animal nutrition in relation to their content in food available in Czech market.

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Deoxynivalenol (DON) and zearalenon (ZEA) are the most frequent *Fusarium* toxins occurring in cereals. When fed to animals they can cause acute as well as chronic toxicoses. According to different studies (Mirocha et al. 1981, El-Hoshy, 1999) they are metabolized after intake and they can be either excreted (urine, breast milk, eggs) or stored in animal tissues (blood, liver, muscles). Thus the food of animal origin might be another way of mycotoxin intake. The aim of this study was to explore the levels of ZEA and DON in cereal and cereal-based complete feed mixes available in Czech market and in particular food of animal origin (milk and eggs) collected in the market during period 2010 – 2012.
Samples were analysed using ELISA RidaScreen ZEA (limit of detection (LOD) = 18 $ng.kg^{-1}$ and limit of quantification (LOQ) = 30 $ng.kg^{-1}$) and ELISA RidaScreen DON (LOD = 1,8 $\mu g.kg^{-1}$ a LOQ = 3,7 $\mu g.kg^{-1}$). About 70% of feed samples were contaminated with both mycotoxins. However, only ZEA was found in tested food samples. Supposedly, ZEA forms major mycotoxin in food of animal origin due to its persistence and fat solubility.

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The work was supported by Ministry of Agriculture (project no. QH81293) and Ministry of Education, Youth and Sports (project COST no. FA 0802)

Co-occurence of Mycotoxins in Feed Produced in the Czech Republic

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EU legislation related to feed aims to guarantee production and distribution of safe feeds, without risk for humans, animals or environment. Directive 2002/32/ES and Recommendation 2006/576/ES indicate Guidance values, that shall not be exceeded. UKZUZ as the Czech National Authority responsible for supervision of feed has checked raw material and finished feed samples from the all steps of the Czech feedstuffs production since 2004.
The presence of the most important mycotoxins in terms of agriculture and animal production was screened. Aflatoxins B_1, B_2, G_1, G_2, Fumonisins B_1, B_2, Ochratoxin A, Deoxynivalenol, Zearalenon and T-2 and HT-2 toxins were determined in maize, wheat, barley, silage, haylage, grass hay, and complementary and complete feedingstuffs. Imported feed such as soya extracted groats, cotton seeds or cocoa hulls were inspected as well. Analysis were performed either by HPLC or LC-MS according to validated and accredited methods.
Basic data on mycotoxins presence in the Czech feed were obtained. The most frequent mycotoxins in all types of Czech feed are DON, ZON and FUMs. The most contaminated groups are complete feedingstuffs, maize and silage. Results confirm that Fusarium mycotoxins are the most frequent contaminants in the CZ feed, although in low levels. Even for these toxins the levels found in feed were lower than EU guidance values.

Mycotoxicological analysis of raw materials as an element of quality control during feed production

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Prevalence of mycotoxins occurrence in connection with present knowledge of their harmful impact on human and animals health becomes an important element of risk identification procedures during production processes. Poland is one of the largest EU poultry meat producers. In poultry production, contaminated feed is the main source of mycotoxins in a production process and therefore quality control strategies should focus particularly on the optimization of feed quality. No EU regulation defining the maximum acceptable concentrations of *Fusarium* mycotoxins in raw materials and feeds, have been established yet. Nevertheless, producers determined to maintain high quality of their products and concerned about the health of the animals, express their interest in the issue of mycotoxins.
The study was carried out in cooperation with a local producer of poultry feed. The mycotoxicological analysis aimed at supporting raw material quality control procedures, thus the decision making process. The study material consisted of cereals harvested in 2011 (40 samples total), randomly collected after preliminary quality evaluation and classification: "wheat – good quality", "wheat – bad quality (suspected of fusariosis)", "wheat contaminated with chaff", "triticale – good quality", "triticale – bad quality (after removing the flour beetle's faeces)", "rye – good quality", "maize – good quality", "maize – bad quality (damaged kernels)", "maize dried using culm", performed by a technologist. Due to the fact that the material was not subject to long-time storage, the study focused on the main *Fusarium* mycotoxins: DON, T-2, HT-2, and ZEA, determined using LC-MS/MS method.
Mean DON and ZEA concentrations in "wheat – good quality" were significantly lower (81.0 and 4.13 ppb) as compared to analogous values in "wheat – bad quality (suspected of fusariosis)" – 966 and 108 ppb, respectively. The contents of other mycotoxins (T-2 and HT-2) in the same two groups were comparable and relatively low (mean values ~1 and 3 ppb, respectively). "Wheat contaminated with chaff" appeared to be the material with the lowest average mycotoxin concentrations: DON – 12.4 ppb, ZEA – 0.80 ppb, T-2 and HT-2 were not detected. Triticale, previously disqualified due to flour beetle presence, contained low levels of mycotoxins: DON – 23.8 ppb, T-2 <0.6 ppb, HT-2 – 2.83 ppb and ZEA – 0.98 ppb). Relatively high contents of mycotoxins were found in "maize – good quality" (mean values: DON – 913 ppb, T-2 – 36.2 ppb, HT-2 – 31.8 ppb, and ZEA – 62.1 ppb). ZEA was present at its highest concentrations in the samples of "maize – bad quality (deformed kernels)" – 106 ppb, on average, while T-2 and HT-2 toxins – in maize dried using culm (mean values 158 and 276 ppb, respectively).
The results of the mycotoxicological analysis were compatible with the results of the technological quality control only partly in case of wheat.

Financial support: "Krok w przyszłość – IV edition" – a PhD grant.

Contamination of DDGS by mycotoxins

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DDGS (Dried Distillers Grains with Solubles) is a balanced by-product from agricultural-food processing, generated as a result of multi-stage grain mash concentration process at the end of the long-term drying stage, purified from alcohol at low temperatures. The USA are the main producer of DDGS's; with the annual production amounting to about 3.2-3.5 million tons. In Europe, its production also increases, and this product is intended for farm animals: poultry, dairy and meat cattle, swine, as well as other carnivores. The interest in the product is quite significant because 1 kg of DDGS contains high proportion of nutrients, particularly total protein (2-3 times as high as in raw material). However in Poland, maize grain harvest is carried out till winter and extremely mouldy material is often used for DDGS production.

The following DDGS samples were subject to mycotoxicological assays: maize (n=30), wheat (n=5), and maize-wheat (n=2) collected from various distilleries in Poland. Trichotecenes and zearalenone were determined applying Bond Elut® Mycotoxin columns. Randomly selected samples were subject to analysis of fumonisins (n=17), aflatoxins (n=10), and ochratoxin A (n=18), to which MultiSep 211 Fum (Romer Labs®), AflaTest® (Vicam), and Ochraprep® (R-Biopharm Rhone Ltd) were respectively used. Mycotoxins were determined using HPLC method (Merc-Hitachi, Agilent) with fluorescent and MS/MS detection. Moulds and yeasts were determined in the selected samples according to PN ISO 7954:1999 with own modifications. Sample cytotoxicity (n=11) was evaluated using MTT test with swine kidney cells.

Results of mycotoxicological analysis of DDGS [ppb]

DDGS	2010/2011	DON	NIV	3ADON	MAS	DAS	T2	HT2	ZEA	FUMO	OTA	AFLA
Maize	No. of samples	30	28	23	23	28	30	30	30	14	15	14
	Positive	30	27	21	10	12	30	30	30	14	8	0
	% of positive	100%	96%	91%	43%	43%	100%	100%	100%	100%	53%	0%
	Mean	1531	36	56	<1,5	<1	27	41	113	184	0,73	nd
	Max	4919	105	177	4,6	3,1	208	279	444	548	3,21	nd
	Min	200	nd	nd	Nd	nd	1,5	78	21,4	10	nd	nd
	Median	566	30	22,4	Nd	nd	14,4	27,5	586	120	<0,4	nd
Wheat	No. of samples	5	5	2	2	2	5	5	5	1	1	1
	Positive	5	2	2	0	0	5	5	5	1	0	0
	% of positive	100%	40%	100%	0%	0%	100%	100%	100%	100%	0%	0%
	Mean	362	12	16,3	Nd	nd	9,3	18,6	25,8	165	nd	nd
	Max	841	30	17,6	Nd	nd	17,3	41,6	68,8	-	nd	nd
	Min	141	nd	15	Nd	nd	6,6	8,8	5,5	-	nd	nw
	Median	267	nd	16,3	Nd	nd	7,5	14,2	23,8	-	nd	nd
Maize-wheat	No. of samples	2	2	2	2	2	2	2	2	2	2	na
	Positive	2	2	2	1	0	2	2	2	2	0	na
	% of positive	100%	100%	100%	50%	0%	100%	100%	100%	100%	0%	na
	Mean	522	4,5	16,9	<1,5	nd	2,0	6,0	37,9	<5	nd	na
	Max	542	4,6	18	-	nd	2,1	6,5	50,3	<5	nd	na
	Min	501	4,5	15,7	-	nd	1,8	5,4	25,4	<5	nd	na
	Median	-	-	-	-	nd	-	-	-	-	nd	na

nd – not detected na – not analyzed

DDGS contained high levels of *Fusarium* metabolites, yet mycological analysis showed only traces of moulds. During the cytotoxicity MTT test, a single sample did not show any cytotoxicity (IC_{50}: 200 mg/ml), while 9 showed low (IC_{50}: 100 mg/ml; 50 mg/ml), and 1 medium (IC_{50}: 25 mg/ml).

Financial support: Ministry of Science and Higher Education Non co-financed international Project No. 761/N – Austria/2010/0

Multi-mycotoxin analysis by HPLC-MS/MS in grain dusts in comparison to analysis in whole grain samples

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Dusts from harvesting and threshing procedures or abrasion during transport are ubiquitous in unprocessed grain lots. They are released during unloading and occur in mills as process dust from winnowing and sieving of the raw material prior to storage and milling. Mycotoxins accumulate on small particles in food or feed bulk. Consequently, dusts are often highly contaminated with numerous mycotoxins that naturally occur in grains. Several multi-mycotoxin methods exist and were validated for wheat, maize, and cereal products. However, none of them was applied on cereal dusts. Hence, the aim of the current study was to develop and validate a multi-mycotoxin method for fast screening of grain dusts.
The developed method comprised a small scale extraction step followed by liquid chromatography with electrospray ionization triple quadrupole mass spectrometry without prior clean-up. More than 30 mycotoxins were detected including A- and B-type trichothecenes, zearalenone, fumonisins, enniatins, ergot alkaloids, ochratoxin A, aflatoxins and alternaria toxins. Limits of detection and quantification, reproducibility, linearity and recovery rates were determined for all analytes in dust samples of wheat, rye, and maize. The influence of matrix effects, mycotoxin accumulation, and sample homogeneity was highlighted by comparing the method performance characteristics as well as time of analysis, required solvent volumes, and determined contents to those of common mycotoxin-analysis procedures applied on respective grain samples. The results might help to evaluate the toxicity of contaminated grain dusts but particularly, are a prerequisite for the development of novel sampling technologies for grain lots such as dust sampling.

Occurrence of mycotoxins in digestate samples of biogas production plants – effect to methanogenesis?

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Production of biogas from animal manure and co-digestion of agricultural waste (and energy crops) is an ecologically worthwhile method to generate energy. Through the anaerobic digestion methane yield is influenced by several factors and should be optimized. One relevant factor is the growth of mould fungi and their production of toxins.
Digestates from real biogas production plants were screened to 30 mycotoxins. A very sensitive and selective method of determination based on high performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS) was applied. Significant amounts of enniatin B, zearalenone, and beauvericin could be detected.
Biogas production will be simulated by batch laboratory tests utilizing animal manure and agricultural waste. Enniatin B, zearalenone and beauvericin will be spiked to the samples as single toxin and as a mixture in the highest concentration measured previously. Untreated samples will act as control. Methane yield will be determined by sampling the biogas produced with a gastight syringe, separation will be performed by gas chromatography.

Acknowledgement
The study was supported by a grant from the Land North-Rhine Westfalia within the EU Ziel2 Programm NRW 2007-2013 (EFRE) for the project "Mykotoxine in Lebens- und Futtermitteln – ein ungelöstes Problem in der Qualitätssicherung".

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Immunochemical studies on the occurrence of *Alternaria* toxins in foods

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Alternaria spp. are ubiquitous plant parasites and may produce a number of mycotoxins, including alternariol (AOH), altenuene (ALT) and tenuazonic acid (TeA). Natural occurrence of *Alternaria* toxins has been reported in various fruits and vegetables.

For the detection of AOH, ALT and TeA, four different EIA systems were used [1,2]. The AOH EIAs were highly sensitive, with detection limits of 35 ± 6.9 pg/mL (monoclonal antibody EIA, mAb) and 59 ± 16 pg/mL (polyclonal antibody EIA, pAb). The ALT and the TeA EIA were also very sensitive with detection limits of 0.23 ± 0.07 ng/mL (ALT) and 5.4 ± 2 n/mL (TeA). Recoveries were variable, but generally acceptable (AOH (pAb): 60-98%, AOH (mAb): 43-80%; ALT: 67-161%; TeA: 34-136%). All tests enabled sensitive detection of the toxins in a low ng/mL or ng/g range.

Apple and tomato products from German retail shops were analyzed. In total, thirty-five samples of apple and tomato products were examined (apple juice 4, tomato juice 15, tomato ketchup 13, tomato paste 3). AOH was found in 91.4% (concentration range 1-5.5 n/mL) of the samples by polyclonal antibody EIA (pAb) and in 80% (concentration range 1-5 n/mL) of the samples by monoclonal antibody EIA (mAb). ALT was found in 34.3% of the samples in a concentration range of 2.45-4.82 ng/mL. TeA was found in 14.3% of the samples in a concentration range of 55-227 ng/mL. Co-occurrence of AOH (mAb and pAb) and ALT was found in 6 samples (17.1%). Co-occurrence of AOH (mAb and pAb) and TeA was found in 4 samples (11.4%) and co-occurrence of AOH (mAb and pAb), ALT and TeA was found in 2 samples (5.7%).

The total daily per capita consumption of fruits and fruit products (including fruit juices) in Germany is approximately 500 g, and vegetables and products thereof (including processed vegetables) account for approximately 235 g per person. Apple and tomato products represent a significant part of these foods. Therefore, AOH, ALT and TeA contaminated products are consumed regularly by the majority of the population. A larger and broader survey is advisable to improve the knowledge about other sources of exposure. Additionally, more toxicological data are necessary to estimate the risk of the *Alternaria* toxin exposure in human health.

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Determination of sensitivity of some apple cultivars against toxin of Isparta isolate of *Alternaria mali*

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In this study the sensitivity of twelve apple cultivars against virulent *Alternaria mali* toxin (A-M toxin) in vitro. Culture filtrate including toxin of *A. mali* were applied on fresh detached leaves. As a result of an artificial inoculation, Summerred, Topred and Golden Reinders were found as sensitive cultivars while Jerseymac, Fuji, Williams Pride and Royal Gala were resistant cultivars to AM toxin.

Entomopathogens as Endophytes

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Entomopathogenic fungi are reported as effective biological control agents against many insect pests of crop plants, and some of these entomopathogens have already been commercialized as biological insecticides. However, use of myco-insecticides applied under greenhouse or field conditions is hampered by several factors and frequently give limited control success. Because of these prevailing problems the idea of testing these EPFs as endophytes came up. Since the seminal studies of Bing and Lewis (1991) many other studies have been published testing the endophytic capacity of *B. bassiana* in plant tissues using different strains and several inoculation methods. In contrast to these studies dealing with endophytic B. *bassiana* isolates, only one paper has demonstrated that *Metarhizium anisopliae* has also an endophytic activity in tomato plants, at the same time promoting plant growth. It is likely that these entomopathogens have more than one mode of action operative in controlling plant pests and pathogens. One of these hypothesized modes of action relates to the production of secondary metabolites, acting as mycotoxins. With regard to this aspect, it is of importance to check whether endophytic strains of *B. bassiana* or *M. anisopliae* produce any of these mycotoxins inside the plants, and in case these accumulate in tissues consumed, would potentially harm consumers' health. We therefore investigated whether strains of *B. bassiana* and of *M. anisopliae* successfully colonize different cultivars of tomato plants either by foliar or seed inoculation, and whether mycotoxins are produced. The amount of biomass of an endophytic fungus in different plant tissues was measured as a proxy for its colonization success using qPCR for pure fungal mycelia and in seed-inoculated plants. Samples from foliar-inoculated plants were subjected to HPLC-MS/MS mycotoxins analyses for Beauvericin from *B. bassiana* and Destruxin A from *M. anisopliae* in tomato leaves and fruits.
Our results demonstrate that *B. bassiana* and *M. anisopliae* strains are successfully colonizing different tomato cultivars with different colonization rates, and this colonization continues until harvesting time. Mycelium is at this time distributed all over the plant tissues, with reduced amount in the newly grown parts, assessed by the recovery rate on a Dodine selective medium, and by qPCR. Two strains of *B. bassiana* and two strains of *M. anisopliae* did not produce mycotoxins inside the plant tissues, while one *B. bassiana* strain showed a low, non-significant production of Beauvericin in the tomato fruits. Additional analyses will focus on other mycotoxins, potentially produced by *B. bassiana* or *M. anisopliae* and their interaction mode within the host plants.

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Relationships between spike and kernel infection and concentration of zearalenone and trichothecenes in grain of winter triticale

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Triticale is a hybrid of wheat and rye created to combine the high yielding capacity of wheat with the stress tolerance of rye, including resistance to diseases, including Fusarium head blight (FHB). There are only few reports discussing the resistance to Fusarium head blight in this cereal. Variation in FHB resistance in triticale is very wide and published data showed high resistance as well as susceptibility of this cereal.
Resistance to FHB of 21 advanced winter triticale lines and 2 cultivars 'Borwo' and 'Fredro' was evaluated. The experiment was conducted under partially controlled environment with use of a mist irrigation. Triticale spikes were inoculated by spraying with spore *F. culmorum* suspension. After inoculation high air humidity was maintained for several days. The first observation was made 10 days after inoculation. Number of infection points per spike was estimated (resistance type 1). The second observation was performed 21 dpi. Number of spikelets affected by the growth of fungus in spike (resistance type 2) was scored. The third observation was made 30 dpi to determine the maximum spike infection (resistance type 7, "late blighting"). After the harvest proportion of *Fusarium* damaged kernels (FDK) and *Fusarium* mycotoxin content in grain were assessed. Zearalenone (ZEA) was quantified using AgraQuant®ZON test. Trichothecenes group B [deoxynivalenol (DON) and derivatives and nivalenol (NIV)] were quantified using gas chromatography technique.
Average number of infection points was 1.1, at a range 0.5-2.3. Number of infected spikelets 21 dpi was on average 4.3, at a range 2.0-8.8. late blighting severity was 10.4, at a range 4.3-16.5 spikelets. Kernel damage by *Fusarium* was high and amounted 47.5%, at a range 15.9-74.8% ZEA, DON and NIV were detected in grain. Only traces of DON derivatives were found. Content of ZEA was 828 ppb. Variation range was wide from 199 ppb to 2005 ppb. Concentration of DON in grain was low and amounted on average 1.295 ppm, at a range 0.369-3.056 ppm. Concentration of NIV was about 6-times higher and was on average 8.761 ppm (4.736-15.586 ppm).
DON amount did not correlate with head infection scores and FDK proportion. Only some positive tendency was observed, except for type 1 resistance. However, significant correlation was found for difference between second and first observation and DON amount. It suggests that DON was produced mainly in early stages of infection. NIV content correlated with type 2 resistance and relationship with FDK was stronger than for DON. Relationships between trichothecenes and late blighting were weak while ZEA concentration correlated significantly with this score.

Reference

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Cis-trans isomerism of zearalenone

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Zearalenone (ZEN) is a mycotoxin produced by *Fusarium* spp. on a variety of cereals. Due to its lipophilicity considerable amounts of ZEN were found in edible oils, especially in maize oil. Toxicological studies revealed estrogenic and teratogenic effects on mammals, thus, EU-maximum levels were established for different foods ranging from 20 µg/kg (infant foods) up to 400 µg/kg (refined maize oil).
Due to the double bond in the lactone ring (C_{11}=C_{12}) ZEN can occur in two isomeric forms, with *trans*-ZEN dominating in nature. The formation of the thermodynamically more stable *cis*-ZEN is induced by light irradiation (fig.1).

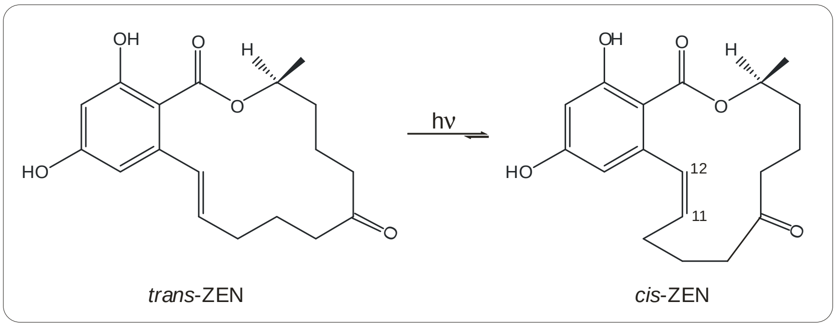

fig. 1: *cis-trans*-isomerisation of zearalenone (ZEN)

The isomers are not or only partially separated by the usual chromatographic methods. Therefore at first a HPLC method was developed that completely separates the two isomers. *cis*-ZEN was produced by UV irradiation of a *trans*-ZEN solution and subsequent preparative purification. Its identity was confirmed by X-ray crystallography [1] and NMR spectroscopy and it was further used as calibration standard for the quantification of *cis*-ZEN in real-life samples.
Investigations on the light-induced isomerisation were carried out showing that *trans*-ZEN is very easily transformed into *cis*-ZEN.
To evaluate if both isomeric forms are present in ZEN-contaminated samples a screening of commercially available maize and hemp oils was performed.

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UV induced *cis-trans* isomerisation of zearalenone in contaminated maize

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The naturally occurring *trans*-ZEN may be transformed by ultraviolet irradiation to *cis*-ZEN. Based on uterotropic assays it was found that *cis*-ZEN exerts a greater affinity to estrogen receptors compared to its *trans* counterpart (Mirocha et al., 1978). However, the practical relevance of this transformation under practical conditions remains unclear. Therefore, the aim of the present investigation was to examine the effect of UV irradiation on the concentration of ZEN in a natural feed matrix.
Two different feed matrices, air dry feed (dry matter (DM) was approximately 89%) and wet feed (DM was adjusted to 33% by adding water), were used. For irradiation of air dry feed, 100g of ZEN contaminated ground maize was weighed in aluminium trays. For irradiation of wet feed, 50g of ZEN contaminated ground maize was weighed in aluminium trays and 85ml water was added. Each of the 4 variants, air dry or wet maize, each irradiated or not irradiated were tested in 5 replicates. The irradiated variant was exposed to UV light (300-420nm) in a special box for 24h, while the control variant was placed covered in the same room for a similar period of time. Moreover, two 5µg/ml ZEN solutions in methanol were prepared. One was stored for 24h in a closed brown glass vessel in the darkness and was used as control. The other one was stored in a closed transparent glass vessel and irradiated for 24h by UV to serve as a positive control for *cis*-ZEN in the high performance liquid chromatography (HPLC) method. Determination of ZEN in maize samples was carried out according to a VDLUFA method (2006) with slight modifications. Analysis of the 5µg/ml ZEN solutions was carried out directly with HPLC without sample preparation after a dilution step.
Both ZEN isomers could be separated with the applied HPLC method, whereby *cis*-ZEN may only be qualified, as no *cis*-ZEN standard is currently commercially available. Neither in the irradiated air dry feed samples nor in all control samples *cis*-ZEN was detected. In the irradiated wet feed samples a 32% lower amount of *trans*-ZEN was determined than in the control samples. Furthermore, a shoulder on the *trans*-ZEN peak was detected and interpreted as *cis*-ZEN, thus the lower amount of *trans*-ZEN was attributed to the conversion to *cis*-ZEN, which however, needs to be confirmed analytically.
In conclusion, the UV induced *cis-trans* isomerisation of ZEN in maize does not seem to be relevant in air dry feed whereas significant amounts may be converted under the investigated wet-feed conditions. The relevance of this finding for practical liquid feeding systems for pigs needs to be determined.

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Screening of total proteins and chitinase enzyme production of *Alternaria mali* isolates of apple

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Introduction
Alternaria species cause diseases in economically important plants. The degredative enzymes produced by plant pathogenic fungi are important in host infection. Production of extracellular enzymes can be detected in culture filtrate and mycelium of fungi. In this study, the protein and chitinase production of three isolates of *Alternaria mali* were investigated.

Material and Methods
A.mali isolates GK-15, GK-31 and 30 K were cultured in liquid medium. Total protein content determined both in culture filtrate and using mycelium of fungus by spectrophotometric analysis (Bradford, 1976). Chitinase activity was assayed in cultural filtrates and grinding mycelium in a buffer, also.

Result and Discussion
Total protein content of isolates GK-15, GK-31 and 30 K were determined as 11.5, 13.4 and 12.5 µg/ml in liquid culture. However, the mycelial extract had the higher total protein content. Total protein content were found 100 fold in grinding mycelium extract and determined as 1600 and 1313.5 µg/ml for 30 K and GK 31 isolates, respectively. The isolates of *A. mali* especially 30 K isolate produced chitinase enzymes in both culture filtrates and mycelium extract.

Total Synthesis of Phase I Metabolites of Alternariol (AOH) and Alternariol-9-methyl ether (AME)

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Alternariol (AOH), a mycotoxin formed by *Alternaria alternate*, was recently reported to act as a topoisomerase poison.[1] Phase I metabolites (Figure 1) of AOH and Alternariol-9-methyl ether (AME) have been detected and identified but not synthesized for further studies or as reference materials so far. These compounds are formed by monohydroxylation of AOH and AME, a prominent metabolic route under cell-free conditions.[2]

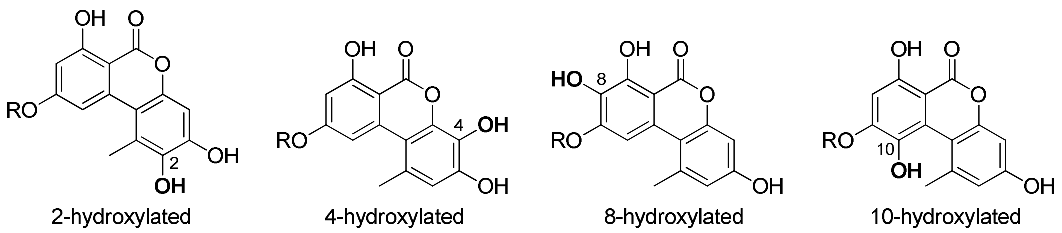
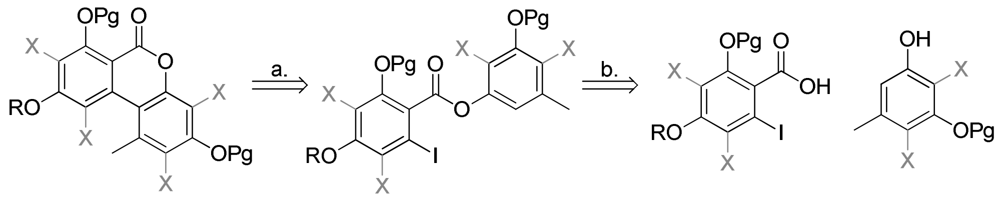


Figure 1: Structures of monohydroxylated metabolites of AOH and AME; R = H (AOH) or Me (AME)

The following strategy, using two key steps (esterification and subsequent C-H-activation) for the formation of the AOH-core structure (Scheme 1), was applied for the total synthesis of the target compounds:



Scheme 1: Key steps of retrosynthetic analysis of target compounds; R = H (AOH) or Me (AME), X = OPg or H, Pg = protective group; a. esterification, b. C-H-activation

Results of the synthesis of different aromatic building blocks and the first monohydroxylated toxins will be presented. Furthermore these compounds will be used for toxicological analysis and serve as reference materials.

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Towards the Synthesis of late Phase II Metabolites of Mycotoxins

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Dihexose and Trihexose-conjugates of mycotoxins (eg. ZEN, DON) are formed in the late phase II detoxification. Due to the lack of reference materials their detection as well as their quantification is still a problem or even currently impossible. Therefore we decided to develop two reliable strategies for the synthesis of such compounds (Fig. 1).

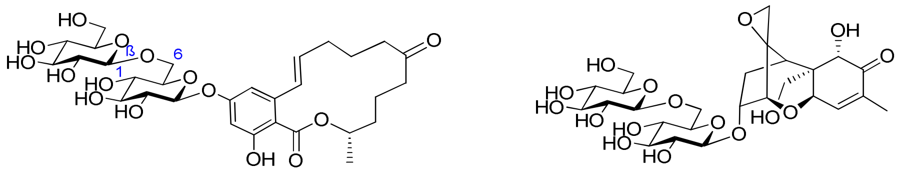

Figure 2: Structure of ZEN- and DON-Diglucoside, respectively

Strategy I starts with Gentiobiose octaacetate, which is converted into a glycosyl donor via insertion of an adequate leaving group. Conjugation with the mycotoxin is planned to be carried out via a Königs-Knorr or N-phenyltrifluoroacetimidate glycosylation approach (Fig. 2).

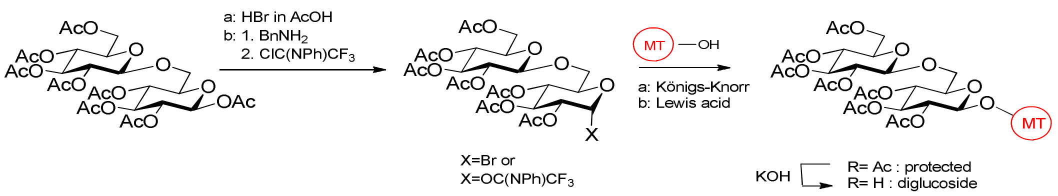

Figure 3: Strategy (I) for the synthesis of diglucosides

Strategy II uses *tert*-butyldimethylsilyl as protecting group for the hydroxylgroup at position 6. This enables the deprotection as well as the conjugation of a further glycosyldonor at position 6 after the conjugation with a mycotoxin. This strategy would open up a pathway to generate a variety of different di- and triglycosides (Fig 3).

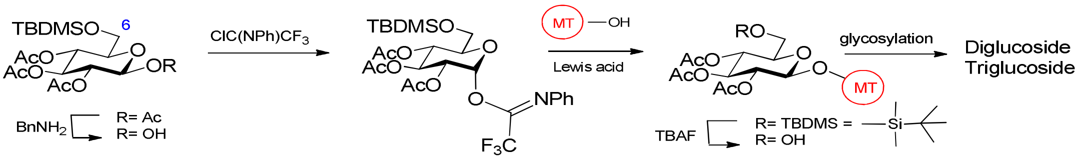

Figure 4: Strategy (II) for divergent synthesis of different mycotoxin conjugates

The synthesis of the glycosyldonors and the conjugation with mycotoxin mimics will be presented within the poster.

Comparison of virulence of *Fusarium avenaceum* isolates in wheat with emphasis on the role of enniatins in plant colonization

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Introduction
Fusarium head blight (FHB), or scab, is an important fungal disease that can occur on all small-grain crops grown, but is seen most commonly on spring and winter wheat, durum and barley. Among *Fusarium* species causing this disease, *Fusarium avenaceum* (Fr.) Sacc., is one of the most important species worldwide. This species can produce significant quantities of toxic secondary metabolites *in vitro*, including enniatins, which are known to be harmful to livestock and pose a safety concern in human food. In this study, nineteen *F. avenaceum* isolates were characterized to their enniatin production in wheat spike and it was focused whether enniatins production affected virulence.

Material and Methods.
Spikes of wheat cultivar Apogee were inoculated with nineteen isolates of*F. avenaceum.* Symptoms of FHB were rated as disease severity at 7, 14, 21 and 28 d after inoculation and as percentage of infected spikelets. Infected wheat spikes were harvested and dried , finely ground and extracted with acetonitrile/water (84/16, v/v). After extraction tenniatins were analysed by HPLC-MS/MS with an 500-MS ion trap (Varian, Darmstadt, Germany). Standards for quantification were prepared by spiking clean meal with known amounts of mycotoxins and extracted in the same way than the samples.

Result and Discussion
Infestation of wheat spikes ranged from8 to 88% of kernels. All isolates of *F. avenaceum* produced enniatins (enniatin A (0,1 to 0,2 µg/g), enniatin A_1 (0,38 to 5,08 µg/g) enniatin B (0,16 to 20 µg/g), enniatin $B_{1.}$ (0,14 to 20 µg/g. Similar virulence profiles on wheat tissue differ between the amounts of enniatins found.

Fusarium poae isolated from cereals grain and associated mycotoxins.

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Fusarium head blight of cereals is common disease occurred all over the world [Bottalico and Perrone 2002]. It caused significant yield loss and deterioration of grain quality as a result of mycotoxins occurrence [Nicholson et al. 2003]. *F. culmorum*, *F. graminearum* and *F. avenaceum* are the main causes of *Fusarium* head blight of cereals. In recent years, significance of *Fusarium poae* increases as a cause of this disease. That species is commonly isolated from grain in cooler climate. Poland is one of the countries where *F. poae* is mostly occurred pathogen responsible for *Fusarium* head blight. It can infect wheat, barley, triticale, oat and rye [Kiecana et al. 2005]. *Fusarium poae* produces mycotoxins such as nivalenol, diacetoxyscirpenol, monoacetoxyscirpenol, scirpentriol, fusarenon-X, neosolaniol, T-2 and HT-2 toxin, beauvericin and enniatin in infected grain [Kulik and Jestoi 2009]. Some authors reported that pathogen produce also deoxynivalenol [Jestoi et al. 2008]. The aim of the study was the determination of toxigenic ability of selected isolates of *Fusarium poae* grew on grain of common wheat and spelt.
In the study 14 isolates of *F. poae* from different cereal species, grew on spelt grain cv. Oberkulmer Rotkorn and Szwabenkorn and winter wheat grain cv. Turnia were used. All tested cultivars before inoculation were not contaminated by any of investigated mycotoxins. Isolates of fungus originated from 4 different cereal species, 8 from oat, 3 from winter rye, 2 from winter triticale and 1 from spring barley. All cereals were cultivated on the organic farms in 2009. The ELISA method was used to identify in infected grain following mycotoxins: deoxynivalenol, T-2 toxin and zearalenone. Four *F. poae* isolates with small round microconidia with base denticale originated from oat, winter rye and winter triticale produced only DON. Five isolates originated from oat, winter rye and winter triticale which have large round microconidia with base denticale were producers of DON and ZEN. Four *F. poae* isolates originated from oat and spring barley with large round microconidia were producers of DON, ZEN and small amounts of T-2 toxins. One isolate from oat with macroconidia produced only T-2 toxin. In presented study, 13 out of the 14, *F. poae* isolates turned out to be the DON producers at amounts from less than 0.25 mg/kg to more than 5 mg/kg with an average of content 1.52 mg/kg.

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Mycotoxin analysis and cytotoxicity of *Fusarium*-inoculated and naturally grown grains

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To study whether cytotoxicity is a suitable functional parameter for the mycotoxin content of unprocessed grains, we compared this relationship in *Fusarium*-inoculated and naturally grown crop samples. Winter wheat (variety Ritmo) and winter triticale (variety Talentro) inoculated with *Fusarium culmorum* and harvested in 2011, and non-inoculated control crops from the same area in Lower Saxony, were kindly provided by Dr. E. Oldenburg (JKI, Braunschweig). Prof. Dehne (INRES, Bonn) provided wheat (variety Ritmo) inoculated with a mix of *Fusarium spec.* and harvested 2010 in North Rhine Westphalia. The mycotoxin content of the samples was analysed by a multi-mycotoxin LC-MS/MS method (Herebian D et al., 2009, J Sep Sci 32, 939-948). With the neutral red assay (Föllmann W et al., 2009, Mycotoxin Res 25, 11-19) the cytotoxicity of extracts from the finely ground grain samples was determined in V79 cell cultures. In parallel, pure reference mycotoxin mixtures comparable in concentration to those identified in the real samples were tested for comparison.
Natural grown wheat and triticale samples from Braunschweig harvested in 2011 had only low mycotoxin contaminations (up to 0.2 µg/g enniatin B and 0.1 µg/g deoxynivalenol (DON)), and extracts from these samples induced no cytotoxicity in the cell cultures. In contrast, a high DON content (up to 32 µg/g), but no enniatin B was detectable in samples from *F. culmorum*-inoculated grains. These samples were clearly cytotoxic for V79 cells. The cytotoxic response induced by the real samples was stronger than that evoked by the reference mycotoxins tested in parallel. That points to other as yet unidentified toxic components in the grain which add to mycotoxin-related cytotoxicity. In normal wheat from Bonn harvested in 2010 very low mycotoxin levels (up to 0.04 µg/g DON and 0.02 µg/g enniatin B) were detected. The inoculated wheat contained somewhat higher levels of up to 1.2 µg/g DON and 0.02 µg/g enniatin B, and the sample extracts induced only weak cytotoxic responses at the highest concentration tested.
In conclusion, naturally grown grain samples from two areas in Germany had rather low mycotoxin contaminations in 2010 and 2011. The *Fusarium*-inoculated grains showed higher mycotoxin contents, and this was also reflected in their cytotoxicity. However, as yet unidentified toxic components in the grain apparently add to the mycotoxin-related cytotoxicity, a finding which is in line with previous studies by us and by others.

Acknowledgement: The study has been supported by a grant from the German state of North-Rhine Westphalia within EU Ziel 2 Programm NRW 2007 – 2013 (EFRE) for the project "Mykotoxine in Lebens- und Futtermitteln – ein ungelöstes Problem in der Qualitätssicherung".

Optimization of deoxynivalenol production in liquid culture by a genetically engineered *Fusarium sulphureum* strain for production of deoxynivalenol-3-glucoside

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High level production of the mycotoxin deoxynivalenol (DON) by *F. graminearum* and *F. culmorum* strains is usually only observed on solid rich media such as autoclaved rice, while production of DON in liquid culture is generally too low for ample purification. In contrast, many type A-trichothecenes such as T-2 toxin and diacetoxyscirpenol (DAS) are produced in high yield in liquid culture by certain Fusarium strains.

We therefore decided to genetically engineer the prolific DAS producer MRC514 (*F. sulphureum*, synonym for *F. sambucinum*) for DON production. First the *TRI13* gene encoding the cytochrome P450, which is responsible for introduction of the hydroxyl group at C4, was knocked out using G418 resistance as selection marker (*tri13*::*nptII*). In order to obtain the hydroxyl groups at C7 and C8 (absent in DAS) the *TRI1* gene from *Fusarium graminearum* was cloned under control of the *pki* promoter from *Trichoderma reesei* and introduced on a plasmid carrying a hygromycin resistance marker (*hph-FgTRI1*) into the previously modified strain. The formation of the keto-group at C8 in type B-trichothecenes is presumably performed by an unspecific oxidoreductase also present in non-trichothecene producing fungi. The resulting transformants were screened for DON production. Besides minor amounts of 15-acetyl-DON (and almost undetectable amounts of 3—acetyl-DON), DON was formed in high yield (up to 800 mg/l) by most transformants. Various parameters such as geometry of culture vessels, fermentation time and temperature were tested. Light was found to highly stimulate DON production.

Since MRC514 is also reported to produce a monoacetoxyscirpenol (MAS)-glucoside (Gorst-Allmann et al., 1985), we tested whether engineering of DON-3-O-glucoside production is possible. A myc-tagged UDP-glucosyltransferase gene from *Arabidopsis thaliana* under control of the CaMV 35S-promoter, was transformed into the engineered DON producing strain using nourseothricin as selection marker (*nat1-AtUGT73C5*). Unfortunately, no D3G could be detected in the resulting strain. However, we observed that transgenic *Arabidopsis* seedlings overexpressing a DON inactivating glucosyltransferase from barley accumulate D3G intracellularly in high yield when treated with DON. The culture filtrate of the engineered *F. sulphureum* strain is a readily available starting material for these experiments.

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Determination of masked fumonisins in three commercial corn hybrids by HPLC-MS/MS technique

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Fumonisins are a group of mycotoxins mainly produced by *Fusarium verticillioides* and *Fusarium proliferatum* and resulted the major contaminants of corn and corn-based products (1). It is well known that these compounds can cause several diseases both in human and animals, in fact the incidence of esophageal cancer in humans has been associated with FB1 exposure (2). Moreover, in the last years the problem of the so called masked mycotoxins has become more and more important, because of the presence of masked mycotoxin forms could improve the real amount of toxicity of a food (3).
Masked fumonisins can be generated by an incorporation of parent fumonisins into macromolecules, such as protein or starch, or by a covalently linkage with more polar compounds. These derivatives can co-exist in the food matrix with their corresponding parent forms and they can be formed directly in plant by fungi, as they represent the result of a detoxifying mechanism, or can be generated by thermal treatment during food processing (4).
In order to better understand the fumonisin masking mechanism, the behaviour of fumonisin occurrence in corn kernels under different conditions was studied in this work. At this aim, three different commercial corn hybrids were chosen: DKC6666, Eleonora and Costanza. The last one was the unique hybrid destined to human consumption. Corn kernels were inoculated with *Fusarium verticillioides* and maintained at different temperatures (20, 25 and 30 °C) and different activity water (0.90, 0.95 and 0.99 a.w.) for two different incubation periods (14 and 28 days). Fumonisin content was determined on both inoculated and not inoculated samples by HPLC-MS/MS analysis. Results showed that fumonisin amounts were hybrid- and temperature-dependent and masked form amounts resulted directly proportional to contamination.

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Monitoring fumonisin A and C production pattern in *Fusarium verticillioides* liquid cultures under different growth parameters

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Fusarium verticillioides and *F. proliferatum* are fungal pathogens able to colonize maize, producing fumonisins as secondary metabolites. Although fumonisins B1, B2 and B3 are the most abundant naturally occurring fumonisins and are usually found in maize and maize-based products, *Fusarium* strains are able to produce other fumonisin analogues, belonging to the series A, C and P. Since these minor compounds occur in traces respect than B-series fumonisins (<5% respect to FB1 in mouldy corn), they have been poorly studied, leading to a lack of information concerning their biosynthetic pathway as well as their production pattern and the environmental conditions able to affect their synthesis.
In this work a selected *F. verticilliodes* strain known as high-producer fumonisins was cultivated on a liquid synthetic media for different incubation periods in two different water activity regimes in order to study the dynamic of the production of fumonisins belonging to the A- and C-series in comparison with the production of B-series analogues. Moreover, since FB4 is the main known precursor of FB1 and FB3, the production pattern of this compound in relation to FB1 has been also monitored with the aim to collect more information concerning the trend of production of the main analogue under different growing conditions.
For these purpose, liquid media previously separated from mycelia were analyzed by using a LC-ESI-MS/MS technique developed in our laboratory, able to allow the simultaneous detection and quantification of twelve fumonisin analogues, demonstrating that biosynthetic study may be suitably supported by advanced analytical techniques.

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Structure Elucidation of New Fusarin Analogs Revealing Insights in the Rearrangement Mechanisms of Fusarin C

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Fusarin C is a *Fusarium* mycotoxin which occurs in corn samples of food and feed (1). Fusarin C is genotoxic and immunosuppressive (2). Former studies showed a mutagenic effect of fusarin C in the Ames test after metabolic activation with the use of S-9 mix (3). Nevertheless, toxicological studies with fusarin C are quite challenging because of the rearrangement of fusarin C due to light and the co-occurrence of further fusarin C analogs. Therefore the rearrangement of fusarin C was examined under reversed phase conditions and under UV-light.
To begin with, the formation of fusarin analogs was monitored under different cultivation conditions. For the first time, the structure of open chain fusarin C was elucidated and based on this data a different structure assignment for fusarin F is proposed. The results were confirmed by detailed NMR measurements and density functional theory (DFT) calculations. In addition, a 15-methoxy-derivative of fusarin C was synthesized to verify the involvement of the 2-pyrrolidone ring in the rearrangement of fusarin C.

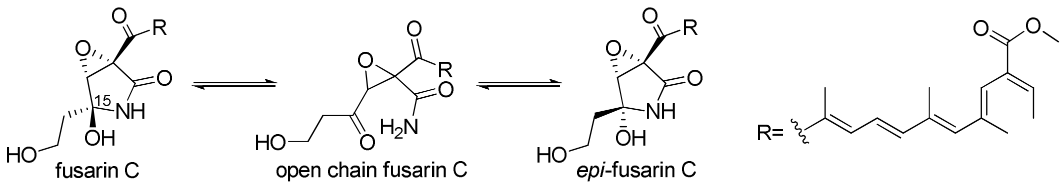

Figure 5: Rearrangement of fusarin C

Furthermore, a new fusarin C like metabolite which was named dihydrofusarin C, was detectable in a crude extract of fusarin C by using high performance liquid chromatography coupled to UV and Fourier transformation mass spectrometry. Due to UV-light, the penatene chain of fusarin C can rearrange to form (10Z)-, (8Z)- and (6Z)-fusarin C. Reference material of the (*Z*)-isomers was obtained and analyzed by HPLC.

We thank Prof. Gelderblom (Medical Research Council, Cape Town, South Africa) for supplying the *Fusarium verticillioides* strain MRC 712.

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Evaluation of qNMR results for purity assessment of crystalline aflatoxin M_1

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Aflatoxin M_1 (AFM1), a major metabolite of aflatoxin B_1 [1], may be present in milk as well as in a wide range of milk-derived products. It is stable during processing, pasteurisation or storage of diary products [2]. AFM1 has been classified in group 1 of carcinogenic agents to humans [3]. To reduce the risk associated with human consumption of contaminated milk, the EU has adopted maximum permissible levels of 50 ng kg^{-1} for AFM1 in milk and dairy products [4].

To support an effective control of the contamination of milk and dairy products by AFM1, IRMM has produced a set of certified reference materials for the determination of AFM1 in whole milk and an AFM1 calibration solution. The latter is currently being replaced by a new calibration solution. IRMM is aiming for a highest quality reference material, including a complete uncertainty estimate and traceability of the certified value to the SI. Particular effort is put on the assessment of the purity of the base material (crystalline AFM1). Several methods were applied: TG-FTIR, tube incineration, HPLC-ELSD, HPLC-DAD, HPLC-FLD, LC-MS as well as qNMR.

In the recent past, quantitative nuclear magnetic resonance spectrometry (qNMR) has been claimed to be the method of choice for purity assessments [5]. Prerequisites to achieve reliable and comparable qNMR results are a well-defined methodological set up, well-characterised standards for internal calibration, careful interpretation of spectral data as well as a fully understood and described uncertainty budget.

Three different laboratories performed independently qNMR measurements. The agreement between laboratories is limited and results are not always equivalent. Taking the results from tube incineration into consideration, the use of different internal standards as well as the possible presence of closely-related homologues like aflatoxin M_2 are plausible explanations for these discrepancies.

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Oxidative stress as one elicitor for the activation of mycotoxin biosynthesis in *Penicillium verrucosum*

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The FAO estimates that up to 25% of the world's crop harvest is contaminated with filamentous fungi or their mycotoxins and must be rejected. For this reason it is necessary to understand the molecular background of mycotoxin biosynthesis in order to develop prevention strategies and to reduce the contamination of food and feed with fungi and their mycotoxins.

In this study, the regulation of the biosynthesis of Ochratoxin A (OTA), a hepatotoxic and nephrotoxic mycotoxin produced mainly by *Penicillium* species like *P. nordicum* and *P. verrucosum* and the biosynthesis of Citrinin, a less potent mycotoxin produced by *P. verrucosum* and *P. citrinum* was investigated during growth under conditions which impose oxidative stress to the fungal cell and could lead further to the generation of reactive oxygen species (ROS).

The mutual regulation of the Citrinin- and Ochratoxinbiosynthesis could serve under this prospect as an adaptation mechanism to compete on different habitats with suboptimal growth conditions. Under oxidative stress conditions like growth on $CuSO_4$ or KNO_2 containing media, but also under light of short wavelength the biosynthesis of OTA is reduced, instead Citrinin is increased. In the typical toxin-producing *P. verrucosum* BFE 808 the intracellular oxidative stress level is reduced under increasing biosynthesis of Citrinin, whereas in non-toxin-producing *P. verrucosum* BFE 613 the intracellular oxidative stress rises relative to the amount of oxidative stress inducing parameters.

Both *P. verrucosum*, BFE 808 and BFE 613, were incubated on MG-media supplemented with $CuSO_4$ (0-40 mg/l) or KNO_2 (0-5 g/l) for 7 days at 25°C. Light-induced oxidative stress was stimulated by incubation on YES under light of 455 nm wavelength for 7 days at 20°C.
The increment of growth was measured, as well as the Biosynthesis of mycotoxins was quantified via HPLC. The intracellular oxidative stress was determined with OxiSelect™ *In Vitro* ROS/RNS Assay Kit (CELL BIOLABS, INC.) relative to biomass.

Citrinin is described to have antioxidative properties (Heider et al., 2005), moreover this mycotoxin is discussed to be light-protective against short wavelength, like UV-light. Here we could show that Citrinin is used as a non-enzymatic protectant against elevated ROS-levels in fungal mycelia.

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Analysis of ochratoxin A in infants' urine and its validation as biomarker of exposure

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Ochratoxin A (OTA) is a nephrotoxic mycotoxin contaminant with a widespread occurrence in food commodities. The exposure of humans to OTA has been documented for several countries, with evident geographical differences. This is reflected in many studies using OTA concentration in blood as biomarker of exposure [1, 2]. But, levels in blood may not characterize a recent exposure as good as urinary OTA levels [3]. Moreover, blood sampling is an invasive procedure not appropriate for certain groups, e.g. infants. Thus, the aim of this work was to develop a non-invasive biomonitoring method, and to validate the OTA-concentration in urine as a biomarker of exposure in infants.

In this work, biological samples were collected from two different cohorts: From the first cohort (Chilean mother-child pairs), milk from nursing mothers (n=50) and urine from their breastfed infants (n=39) were collected. The second cohort (Turkish infants) provided urine samples (n=30). OTA analysis of all samples was done using previously validated methods which involve liquid-liquid extraction with chloroform followed by LC-MS/MS analysis [4].

OTA was detectable in most of the milk and urine samples. The highest values were observed in neonates, and lower levels were observed at later stages of breastfeeding. The comparison of individual pairs of values (n=27) for OTA intake and urine levels showed a rather good correlation (r=0.57) for this type of studies. When urines of Turkish infants were analyzed, it was evident that OTA exposure of this cohort was considerably higher than that of Chilean infants. A more detailed analysis of OTA metabolites revealed the presence of OTA conjugates (glucuronide/sulfate) in highly exposed Turkish infants, but not in urines of Chilean babies. These results indicate that OTA-conjugation may represent an important metabolic pathway, yet only at high exposure levels.

OTA concentrations in urine reflected rather well the dietary exposure of infants calculated from breast milk. This work is the first demonstration that OTA concentration in urine is a valid biomarker of exposure for infants.

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Glutathione Conjugates Of Ochratoxin A As Biomarkers Of Exposure

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Ochratoxin A (OTA) is a mutagenic mycotoxin classified as a possible human carcinogen that undergoes metabolism to generate reactive electrophiles that react with sulfhydryl groups and DNA bases to form covalent DNA adducts (Pfohl-Leszkowicz & Manderville, 2007; 2012). In the present study we have utilized the photoreactivity of OTA to generate authentic samples of reduced glutathione (GSH) and *N*-acetylcysteine (NAC) conjugates of the parent toxin. These conjugates along with the nontoxic OTalpha, which is generated through hydrolysis of the amide bond of OTA by carboxypeptidase A, were utilized as biomarkers to study the metabolism of OTA in the liver and kidney of male and female Dark Agouti rats. Male rats are more susceptible than female rats to OTA carcinogenesis with the kidney being the target organ (Pfohl-Leszkowicz et al, 1998; 2002). Our studies show that the distribution of OTA in male and female rat kidney is not significantly different. However, the extent of OTA metabolism was greater in male than female rats. In the liver much higher levels of OTalpha were detected compared to the kidney and formation of OTalpha is a detoxification pathway for OTA. In addition the metabolites excreted by male and female are different. The female excreted faster and much more metabolites than male. These findings suggest that differences in metabolism between male and female rat provides an explanation for the higher sensitivity of male rats to OTA toxicity.

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Mycotoxin mixtures: Studies on the individual and combined toxicity of citrinin and ochratoxin A in cultured cells

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Mycotoxin contaminations in foods and feed are of concern since they may cause adverse effects on animal and human health. Ochratoxin A (OTA) and citrinin (CIT) are common contaminants of crops and can occur together in various food commodities. Both are known to exert nephrotoxicity in different species and show genotoxic properties in cells. In light of possible combined exposures it was the aim of the present study to investigate, whether cytotoxic and genotoxic effects of OTA and CIT are enhanced by combination of both mycotoxins as compared to their individual effect.
Cell viability, as determined by the neutral red uptake assay in V79 cells [1] after exposure for 24h, showed a concentration-related response. The IC_{20} and IC_{50} values for OTA were 10 and 25 µM, those for CIT were 70 and 100 µM, respectively. When cells were simultaneously treated with concentrations of OTA and CIT which alone showed low to intermediate cytotoxicity, additive effects were observed. Combinations at higher concentrations resulted in over-additive cytotoxic effects. But, this was not seen with mixtures at low concentrations where the individual components elicit no response. Similar results were observed in the micronucleus (MN) genotoxicity assay [2]: We found an increase of MN with either OTA or CIT at slightly cytotoxic concentrations, and additive effects of their mixtures at low micromolar levels. Combined effects of OTA and CIT at higher concentrations were clearly of synergistic nature.
In addition we investigated the origin of the MN by using a human antibody (CREST) against the centromere/kinetochore complex. At low cytotoxic concentrations, OTA induced predominantly CREST-negative micronuclei. In contrast, mainly CREST-positive micronuclei were detectable above 10 µM of OTA. Unexpectedly, CIT induced mainly CREST-negative micronuclei under these treatment conditions.
Our results show that cyto- and genotoxic effects of OTA and CIT mixtures *in vitro* clearly depend on the test concentrations: Combinations at lower micromolar concentrations exert additive effects, and only rather high and toxic concentrations induce over-additive effects. However, these concentrations are far higher than blood levels found *in vivo* in healthy persons with dietary mycotoxin exposure.

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The study has been supported by a grant from the German Federal Land North-Rhine Westphalia within EU Ziel 2 Programm NRW 2007 – 2013 (EFRE) for the project "Mykotoxine in Lebens- und Futtermitteln – ein ungelöstes Problem in der Qualitätssicherung".

An air-liquid interface (ALI) culture of porcine intestinal epithelial cells (IPEC-1) promotes morphological differentiation and increases the sensitivity to deoxynivalenol

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The intestinal barrier is established and characterised by the interplay of different cellular and molecular components. The luminal border of the intestinal barrier consists of a polarised epithelial monolayer. These cells are responsible for digestion, selective uptake, the transport of nutrients from the luminal side of the gut to the blood and build a barrier for pathogens and toxins. On the other side there are close contacts called tight junctions between adjacent epithelial cells. The tight junctions prevent an uncontrolled paracellular passage of molecules and microorganisms. These functional requirements on the epithelial cells imply a polarised morphology and a three-dimensional localisation of transport- and enzyme systems. The establishment of a suitable epithelial cell culture model has to fulfil these preconditions. The aim of this study was the establishment of an *in vitro* cell culture system which resembles the intestinal barrier *in vivo* as closely as possible.
The air liquid interface (ALI) culture is the method of choice in the culture of airway and gastric epithelial cells. In the ALI cultures the apical medium is reduced to a thin film, which results in an improved oxygen supply. In this study, the analysis of important morphological and functional features was realised through different methods and the comparison of the three culture conditions: impermeable support, permeable support and ALI-culture.
Cultured cells were examined by immunfluorescence and electron microscopy. We analysed cryo sections of the gut with the same methods to compare *in vitro* with *in vivo*.
In comparison of the different culture conditions a significant increase in the number of cells, the thickness of the layer and in the accumulation of Zo-1 by cell-cell-contacts were detected. In contrast, no differences between ALI and *in vivo* could be observed. A clear decrease of the calculated volume of the cells from dish-cultivation to ALI was noticed reflecting in ALI-cultures the conditions close to the physiological situation.. By electron microscopy, differentiation of cells was detected in a change from flat and long to a high prismatic phenotype of cells.

The mycotoxin deoxynivalenol caused a significant TEER-decrease in the ALI cultures after 24h. In conventional membrane cultures a significant TEER-decrease was found after 48h. Obviously IPEC-1-ALI-cultures show not only a typical intestinal epithelial morphology but are also more sensitive to deoxynivalenol than conventional membrane cultures.

This is the first report of an air-liquid interface culture of intestinal epithelial cells, suggesting that an air-liquid interface is functioning as an important factor in the morphological differentiation of intestinal epithelial cells *in vitro*.

In vitro model on immunotoxic effects of deoxynivalenol in murine lymphoblasts

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Contradictory results have been reported on the immunotoxic properties of deoxynivalenol (DON). Studies on its immunotoxicity have been mainly conducted in mouse and chicken models, in which DON was shown to suppress the antibody response to vaccine. However, it is difficult to elucidate the basic molecular and cellular mechanisms of these immunotoxic effects in animal models. Thus we intended to introduce a cell culture model and examined in an in-vitro study the effects of DON on the cell growth of the murine lymphoma cells.

Murine YAC-1 lymphoma cells (ATCC #: TIB-160; Mo-MuLV- induced lymphoma lymphoblasts) at an initial concentration of 1 x 10^5 cells/ml were employed as an easily applicable in vitro model because lymphocytes have repeatedly been shown to be sensitive to immunotoxicity. Cells were quantified using a Neubauer chamber and cell viability was assessed via trypan blue exclusion. The blastogenic activity of lymphoblasts was measured by a proliferation test and incorporation of tritiated thymidine. Cultures were harvested on glass fiber filters and ^{3}H activity was measured by a scintillation analyzer.

The total counts of surviving YAC-1 cells were dose-dependently significantly lower ($p \leq 0,005$) in samples incubated for 48 hours with increasing concentrations of DON (2.08 ± 0.34, 0.33 ± 0.09 and 0.25 ± 0.10 x10^6 /ml; respectively) compared to control cells (3.02 ± 0.36 x10^6 /ml). In confirmation of these results also the cell proliferative activity, measured by thymidine incorporation, was dose-dependently five to ten times lower in YAC-1 cells incubated for 48 hours with DON in comparison with controls.

These results let suggest that YAC-1 lymphoma cells are a suitable and easily applicable model to investigate and elucidate the basic molecular and cellular mechanisms of the immunotoxic effects of DON. This in-vitro model enables to study potential prophylactic and therapeutic effects in-vitro before or even without any costly animal experiments.

A yeast-based chemogenomic approach to explore cellular effects of Deoxynivalenol toxicity.

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The *Fusarium graminearum* toxin Deoxynivalenol has been shown to inhibit eukaryotic translation. The immediate consequences of toxin exposure on a cellular level have not been investigated in a systematic manner. We use the yeast *Saccharomyces cerevisiae* as a model system to investigate the different levels of Deoxynivalenol effects on a simple eukaryote. The isolation of DON resistant alleles in genes coding for ribosomal proteins demonstrates a similar mode of action of DON in yeast. We use a chemogenomic assay using all available mutant strains covering the nonlethal deletion mutants and a range of temperature sensitive mutants. To facilitate the susceptibility screens we deleted the gene coding for the major drug efflux pump Pdr5 from all strains. Growth of the mutant strains on solid medium supplemented with DON reveals a number of cellular secondary targets of DON. Immediate responses are being investigated by transcript profiling. We expect that the synthesis of these two approaches will allow us to pinpoint the protective responses of a cell to exposure of a fungal toxin.

Effects of T-2 Toxin and HT-2 Toxin on the Blood-Brain Barrier *in vitro*

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The mycotoxin T-2 toxin is a trichothecene of the type A subgroup produced by fungi of the *Fusarium* species affecting various cereals. The main T-2 toxin metabolite in different *in vivo* as well as *in vitro* studies was characterized as HT-2 toxin, the deacetylation product of T-2 toxin at the C-4 position [1; 2]. Furthermore, T-2 and HT-2 toxin often occur together in infected cereals or processed food samples.
Concerning the toxicity, several acute as well as chronic effects could be observed in animal studies after T-2 toxin exposure. Experiments regarding the nervous system reported an alteration of the brain neurotransmitter concentrations after dietary exposure to T-2 toxin [3]. An increase in the blood-brain barrier (BBB) permeability was observed in mice after percutaneous exposure to T-2 toxin [4]. However, data on the effects of T-2 toxin and its main metabolite HT-2 toxin on the BBB and the central nervous system are rather limited.
In this study, the BBB was mimicked by using porcine brain capillary endothelial cells (PBCEC) seeded on polycarbonate filter systems. As an indicator for endothelial barrier function the transepithelial electrochemical resistance (TEER) was measured using a CellZscope®. First experiments on the cytotoxicity of T-2 and HT-2 toxin on PBCEC showed cytotoxic effects in the lower nanomolar range. A total disruption of the barrier function was observed in nanomolar concentrations in a time dependent manner for both tested toxins. The loss of barrier integrity can result in the disintegration of the intercellular tight junction protein occludin. Immunocytochemical analysis of occludin using fluorescence microscopy revealed the loss of the tight junction protein after application of T-2 toxin. Further studies are currently under way to characterize any possible transport of the toxin and its main metabolite across the BBB *in vitro*.

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Formation, genotoxicity and inactivation of zearalenone catechol metabolites

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Zearalenone (ZEN) is an estrogenic mycotoxin produced by *Fusarium* species. The adverse effects of ZEN and its reductive metabolite alpha-zearalenol (alpha-ZEL) are often compared to those of 17beta-estradiol (E2) and estrone (E1). These endogenous estrogens are associated with an increased risk for cancer, which may be mediated by two mechanisms, i.e. (i) hormonal activity and (ii) genotoxic effects by cytochrome P450-catalyzed metabolic activation to catechols (Wang *et al.*, 2010). Like E2 and E1, ZEN and alpha-ZEL exhibit marked estrogenicity and also undergo aromatic hydroxylation to catechol metabolites (Pfeiffer *et al.,* 2009). The subsequent methylation of catechols by catechol-*O*-methyltransferase (COMT) is generally considered to be a detoxifying pathway. Imbalances between these activation and inactivation reactions can lead to the formation of reactive semiquinones which can alkylate DNA or produce reactive oxygen species by redox cycling.

The genotoxicity of the catechol metabolites was determined in a cell free system by measuring 8-oxo-2'-deoxyguanosine using a LC-MS/MS method. The individual catechols of ZEN, alpha-ZEL, E2 and E1 are able to induce oxidative DNA damage in calf thymus DNA. The ranking of the DNA damaging activity was 15-HO-ZEN > 2/4-HO-E2/E1 > 15-HO-alpha-ZEL > 13-HO-ZEN/alpha-ZEL. When hepatic microsomes from different species were incubated with ZEN , the rat was found to have the highest activity for catechol formation, followed by human, mice, swine and steer. The amount of catechol metabolites correlated directly with the amount of oxidative damage in calf thymus DNA. Concerning the detoxification pathway via methylation by human hepatic COMT the ranking was 2-HO-E2/E1 >> 4-HO-E2/E1 > 13/15-HO-ZEN/alpha-ZEL.

The mycoestrogen ZEN and its reductive metabolite alpha-ZEL undergo metabolic activation to reactive catechols with a DNA-damaging potential, in analogy to the steroidal estrogens E2 and E1. Compared with the steroidal catechol estrogens, all catechol metabolites of ZEN and alpha-ZEL are poorer substrates for human COMT and some exhibit an even higher reactivity towards DNA.

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Supported by Deutsche Forschungsgemeinschaft (Grant ME 574/32-1) and "Food and Health" of KIT.

Alternariol induces oxidative Stress without further damage to DNA bases

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Fungi of the genus *Alternaria* are widespread contaminants and relevant to the spoilage of food and feed (Li a. Yoshizawa, 2000). Exposure to extracts of *Alternaria spp*. has been associated with enhanced incidence of esophageal cancer (Liu et al., 1991). Alternariol (AOH) and AME, two of the main mycotoxins of *A. alternata*, are known to exhibit genotoxic potential, whereas the mechanism of action is not completely revealed yet (Fehr et al., 2009). In the present study we addressed the question whether AOH and AME induce the formation of reactive oxygen species potentially contributing to their genotoxic properties

In the dichlorofluorescein (DCF) assay a concentration-dependent increase of reactive oxygen species was observed in HT29 colon carcinoma cells after 1 h of incubation with AOH. AME induced ROS as well, however to a minor extent. The amount of produced ROS by AOH increased up to 250 %, whereas in parallel the tGSH-level transiently decreased slightly after 1 h of incubation. In accordance with the results of the DCF-assay an increase of nuclear Nrf2 was observed, indicative for the onset of oxidative stress. As a potential target gene of Nrf2-signaling the activity of the glutathione S-transferase (GST) was determined, showing a slight but significant increase (110 % at 50 µM AOH after 24 h of incubation). The induction of ROS by AME however did not affect GST activity. The results of the comet assay indicate an increase in DNA-strand breaks by AOH after 1h of incubation, but no induction of fpg-sensitive sites.

Taken together, incubation of HT29 cells with AOH has been found to generate ROS and activate the redox-sensitive signalling Nrf2 pathway, but did not induce oxidative DNA damage. Thus oxidative stress induced by AOH and AME appears not to contribute substantially to the genotoxic properties of the mycotoxins.

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Impact of *Alternaria* toxins on CYP1A1 expression in human tumor cells of different origin

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Mycotoxins of the genus *Alternaria* are widespread contaminants of food and feed comprising a potential risk for health. Alternariol (AOH) and alternariol monomethylether (AME) have been reported to possess genotoxic properties, inducing enhanced levels of DNA damage. Recent studies indicate, that AME and AOH are metabolized by cytochrome P450 monooxygenase enzymes (CYPs), preferentially by CYP1A1 (Pfeiffer *et al.*, 2008), either to catechols or hydroquinones, indicating that the oxidative metabolism of these mycotoxins may also be of toxicological relevance. In the present study two questions were in focus: 1) whether and to what extend *Alternaria* toxins induce CYP1A1 expression and whether toxin-mediated CYP1A1 induction is aryl hydrocarbon receptor (AhR)-dependent and 2) in this context do *Alternaria* toxins show organ selectivity.
In cells of different origin: colon (HT29), esophagus (KYSE510) and liver (HepG2) incubated for 24 h with AOH or AME comparable cytotoxic effects of the mycotoxins were observed at a concentration of 50 µM. Both mycotoxins significantly induced the expression of CYP1A1 as measured by mRNA levels and enzyme activity. In HepG2 and KYSE510 cells the maximum of induction was observed at 10 µM for both *Alternaria* toxins. Esophageal cells responded more sensitive to the mycotoxins than liver and colon tumor cells, which showed no induction of CYP1A1. The results indicate that cells of different origin may respond differently to AOH and AME in context with CYP1A1 induction, thus hinting at an important role of the biotransformation of the mycotoxins for toxicity. The role of the aryl hydrocarbon receptor signalling in the mycotoxin-mediated induction of CYP1A1 in human esophageal carcinoma cells was investigated by siRNA technology. In AhR-suppressed KYSE510 cells the CYP1A1 induction of AOH and AME was almost abrogated, indicating dependence on the transcription factor and supporting the results of Schreck et al. (2011) in murine hepatoma cells. The results allow to hypothesize that AhR-dependent induction of CYP1A1 by AOH and AME potentially contributes to the toxic properties of the *Alternaria* toxins.

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Development of an improved *in vitro* digestion model – influence on apparent zearalenone content in flour

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Background: Oral bioavailability of a compound can be seen as the result of three processes: 1. Bioaccessibility i.e. release of the compound from its specific matrix into digestive juice in the gastrointestinal tract; 2. Intestinal resorption; 3. Metabolism of the compound [1]. Particularly the first issue also applies to contaminants like mycotoxins from food and feed. However, for risk assessment these effects are often neglected. An option to include digestion effects is the application of in vitro models that mimic the release of a toxin from its distinct matrix. Such assays have been developed and applied for contaminants from soil (DIN 19738) and also for mycotoxins from foods [1]. A serious drawback of the models presented so far is the lack of verification for the activity of the various digestion enzymes throughout the assay runtime. E.g. changes in pH-value or old/inactive enzymes would hamper to obtain correct data, as no positive control can be implicated in the digestion model.
The impact of digestion on the amount of bioaccessible mycotoxins was shown for the fumonisins earlier. Hidden forms of the toxin can be present in foodstuff that remain undetected by classical extraction methods, however, they can be released by in vitro digestion [2].
Objective: The aim of this work was to establish a protocol that enables an "at line"-verification of the activity of enzymes used in an in vitro digestion model. As a practical application the developed in vitro digestion model is used for the investigation of zearalenone (ZEN) contaminated flour. The results are compared to data derived from conventional ACN/H_2O extraction and LC-MS/MS-analysis.
Results: Four photometric assays were established that allow an "at line"-verification of the activity of the crucial enzymatic steps (alpha-Amylase, Pepsin, Trypsin, and Lipase) at the different stages of the digestion process. A sampling directly from the incubation and transfer into the assay is feasible without interference with the digestion process itself.
ZEN is completely stable under digestion conditions as it could be shown by a recovery of approx. 100% for ZEN-spiked blank matrix. Further it was shown that in vitro digestion of flour samples led to ZEN values that were up to 50% higher than those values obtained after classical ACN/H_2O extraction.
Conclusion: The presented assays provide a simple opportunity to monitor the enzymatic functionality of the in vitro digestion model. Our data derived from the use of the digestion assay show that the classical ACN/H_2O extraction used for the analysis of ZEN is prone to underestimate the content of bioaccessible ZEN in naturally contamined flour.

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Effects of deoxynivalenol (DON) and lipopolysaccharide (LPS) on liver histopathology and blood chemistry in pigs

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Deoxynivalenol (DON) is a *Fusarium* derived mycotoxin and is frequently detected in toxicologically relevant concentrations in feed. Monogastric animals like pigs are sensitive regarding DON. Additionally the gastro-intestinal tract represents a natural source of LPS and the liver as the important metabolic and excretory organ is involved in the detoxification of gut derived substances. As a synergistic effect of combined administration of both DON and LPS is hypothesised, we studied the impact of DON feeding and the intravenous (i.v.) administration of DON and/or LPS on liver histopathology (n=39) and liver enzymes (n=37).
Six groups were used: (1) control diet + saline i.v.; (2) control diet + 100 µg/kg BW DON i.v.; (3) control diet + 7.5 µg/kg BW LPS i.v.; (4) control diet + 100 µg/kg BW DON i.v. plus 7.5 µg/kg BW LPS i.v.; (5) oral DON diet (3.1 mg/kg) for 5 weeks + saline i.v. and (6) oral DON diet (3.1 mg/kg) for 5 weeks + 7.5 µg/kg BW LPS i.v.. Saline, DON and/or LPS were given i.v. for 60 min, beginning 3 h prior to slaughter. Blood samples were taken in an interval of 30 to 60 min until slaughter and examined for serum albumin, aspartate-aminotransferase (AST), bilirubin, gamma-glutamyltransferase (GGT), glutamate dehydrogenase (GLDH) and total protein. After slaughter HE stained paraffin sections were prepared from the liver and the lesions were microscopically scored on blinded sections. The Histology Activity Index (HAI) was used to enable a comparison between criteria (portal, periportal and acinar inflammation of neutrophilic and eosinophilic granulocytes, focal lytic and confluent necrosis, haemorrhage) (Ishak *et al*. 1995). We did not detect an effect of DON feed on blood chemistry, whereas infusion of DON and LPS, alone or combined, did show a marked impact. Compared to control, LPS alone (group 3) showed a significant decrease in albumin (control vs. group 3 [g/L]: 28.5±1.6 vs. 24.7±0.9). Total protein decreased after LPS (group 3) and DON/LPS administration (group 4) at 180 min (control vs. group 3, 4 [g/L]: 58.8±2.0 vs. 51.5±0.9, 51.4±1.5), whereas in these two groups bilirubin increased pathologically at 180 min (control vs. group 3, 4 [µmol/l]: 1.0±0.2 vs. 5.4±1.0, 8.3±1.4) as an indicator for cholestasis and hepatocellular injury. AST, GGT and GLDH were not affected. An increased total HAI was found for LPS, alone and combined with DON, which revealed a significantly detrimental effect on liver morphology (control vs. group 3, $p \le 0.05$, control vs. group 4, $p \le 0.01$). This was also reflected in individual criteria such as portal and acinar inflammation and haemorrhage. No effects on HAI were found for DON feed and infusion with LPS (group 6).
In conclusion, LPS showed a marked detrimental effect on liver morphology and function, whereas DON did not damage the liver. Furthermore, DON feed seems to alleviate LPS effects. In the end the hypothesized synergistic effect of DON and LPS could not be supported by the present study.

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DOM-1 does not affect the cell performance of porcine PBMCs

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Deoxynivalenol (DON), a trichothecene produced by *Fusarium* species, is frequently found in animal feed. This mycotoxin is known to induce vomiting, decrease feed intake, and to affect the immune system, particularly in pigs. Therefore, the effects of DON are a major economic problem yielding high costs every year. Inhibition of protein synthesis and induction of apoptosis on a cellular level are considered the main mechanisms of its toxicity. Therefore, it is often suggested that tissues with a high protein turnover or quickly proliferating cells, such as immune cells like peripheral blood mononuclear cells (PBMCs), respond sensitive to DON. DON is bio-transformed by the bacterial rumen strain *Eubacterium* BBSH to the non-toxic de-epoxy-DON (DOM-1). DOM-1 differs to DON in lacking the 12,13-epoxy ring. The de-epoxidation by the BBSH 797 by opening the ring is a detoxification reaction. The effect of DON and DOM-1 on proliferation was analysed for porcine PBMCs via a BrdU incorporation test. DON showed a decrease in proliferation at concentrations from 250 ng/mL onwards. A direct comparison of DOM-1 with DON revealed that DOM-1 was 400 times less proliferative than DON (250 vs. 100,000 ng/mL, respectively) (Figure 1). The less-proliferative effect of DOM-1 cannot be explained by a decreased stability, as DOM-1 was retrieved in the LC-MS/MS, proving that DOM-1 is stable in the course of the experiment. Data confirmed that DOM-1 does not cause the same negative effects as DON.

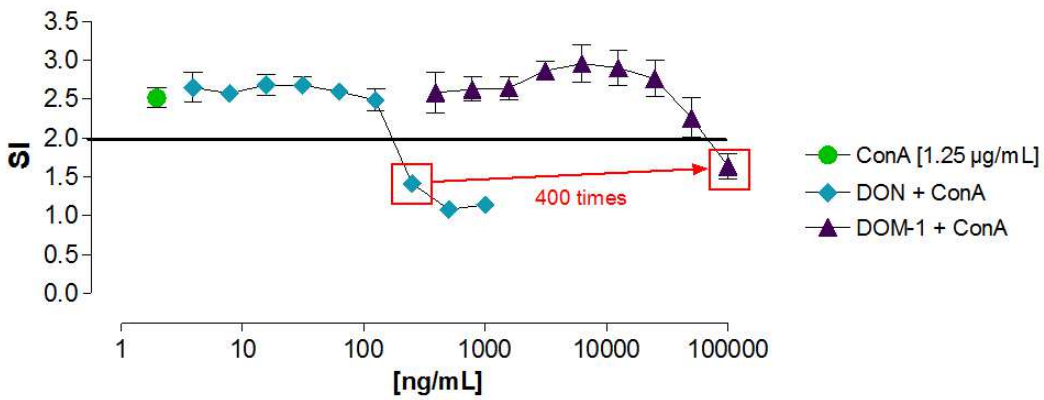

Figure 1
A serial dilution of DON + ConA and a 100 times higher concentration of DOM-1 + ConA was analysed using a BrdU assay.
SI: stimulation index; ConA: concanavalin A; DON: deoxynivealenol; DOM-1: de-epoxy-deoxynivalenol

Amelioration of OTA induced immunotoxic effects by silymarin and vitamin E in chicken

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Objectives: Silymarin (SL) is the bioactive extract of a plant named *Silybum marianum* while vitamin E (VE) is an important naturally occurring antioxidant. The present study was designed to evaluate the ameliorative effects of silymarin and vitamin E against ochratoxin A (OTA) induced immunotoxic effects.

Materials and methods: One day-old White leghorn cockerels divided into 12 groups each having 20 birds were kept on feeds amended with different combinations of OTA1 & OTA2 (1.0 and 2.0 mg/kg), SL (1.0 g/kg) and VE (200 mg/kg). Different dietary treatments / groups were as follows: i. Control, ii. OTA1 only, iii. OTA2 only, iv. SL only, v. VE only, vi. SL+VE, vii. OTA1+SL, viii. OTA2+SL, ix. OTA1+VE, x. OTA2+VE, xi. OTA1+SL+VE and xii. OTA2+SL+VE. Duration of the experiment was 42 days. Birds were sacrificed at 21 and at 49 days of age. Organ weight and pathologic studies were performed. Different immunological assays including lymphoproliferative response to avian tuberculin, mononuclear phagocytic index as determined by carbon clearance assay and antibody response to sheep red blood cells (SRBC) were performed.

Results: Groups kept on feeds contaminated with OTA (1.0 and 2.0 mg/kg) alone showed a significant decrease in relative weight of bursa of Fabricuis. Feeding of SL and or VE along with 1.0 mg/kg OTA rendered this difference non significant. This amelioration was however, not observed when SL and or VE were fed along with 2.0 mg/kg OTA. Lymphoproliferative response to subcutaneous injection of tuberculin, phagocytic index of circulating macrophages and antibody response to SRBCs were significantly lower in groups fed 1.0 and 2.0 mg/kg OTA compared with control birds. A concurrent feeding of SL and or VE with 1.0 mg/kg OTA rendered these differences non significant from the control. This improvement in immune responses was, however, not observed in birds fed SL or VE with 2.0 mg/kg OTA.

Conclusion: SL and VE possessed the ability to ameliorate the OTA induced immunotoxic effects in chicks. This amelioration was, however, observed only in chicks fed 1.0 mg/kg OTA and not in those fed 2.0 mg/kg OTA.

Impact of Physicochemical Parameters on the Decomposition of Deoxynivalenol During Extrusion Cooking of Wheat Grits

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The mycotoxin Deoxynivalenol (DON) is found in cereals and cereal products as most processing techniques lead only to a partial reduction of deoxynivalenol levels. One technique with a reported relatively high impact on deoxynivaleol decomposition is extrusion cooking. In the current work, systematic studies of a range of physicochemical parameters such as temperature, moisture, compression, residence time in the extruder, pH value and protein content on their impact on deoxynivalenol decomposition during extrusion cooking were performed. The analysis of deoxynivalenol was made by HPLC-MS/MS using a QuEChERS based cleanup with 15-d_1-deoxynivalenol as internal standard. It could be shown that the reduction of deoxynivalenol levels is dependent on a set of parameters partially interacting with each other. Especially moisture content and compression are key factors for the reduction of deoxynivalenol levels. A correlation between residence time of the mycotoxin in the extruder and deoxynivalenol degradation was also observed when screws without a compression factor were used. Generally, the reduction of deoxynivalenol levels was increased by the use of screws with a high compression factor. As known from cooking, deoxynivalenol could also be easily degraded by extrusion under alkaline conditions. Furthermore, an increase of the protein content of the starting material resulted in higher reduction rates of deoxynivalenol.

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Acknowledgement

This study was supported by a fellowship to Q.W. by China Scholarship Council. We also thank the state of North-Rhine Westphalia and the European Union in the Ziel 2-Programm NRW for funding.

Degradation of deoxynivalenol and its derivatives by microorganisms contained in a commercial dry sourdoughs

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Biological methods such as using microorganisms to degrade mycotoxins are one of the safest ways to protect food products against mycotoxins. The bacteria of lactic fermentation can be effectively used to degrading mycotoxins. The aim of this study was to determine the effect of the conduct of dough fermentation with using different sourdoughs on the content of nivalenol, deoxynivalenol and its derivatives in the dough made from contaminated rye flour.
The material for research were commercial sourdoughs, one strain of *Lactobacillus fermentum,* and rye flour type 750 artificially contaminated with mycotoxins in the amount of 1mg*kg^{-1}.
The dough was prepared with rye flour and examined dry sourdogh and fermented for 12 and 24 hours at 32°C. After fermentation, samples were frozen and then dried in a lyophilizer LABCONCO FREEEZONE. Dry samples of dough after grounding were cleared according the SPE method (Valle-Algarra et al. 2005) and analyzed on presence of mycotoxins: deoxynivalenol, 3-acetylodeoxynivalenol, 15-acetylodeoxynivalenol and nivalenol, using gas chromatography coupled with MS detector (GC- MS QP 2010 PLUS, SHIMADZU) and capillary column Factor Four: VF-1 ms,: 30m; 0,32 mm; IDDF= 0,25 µm.
The results of degradation of mycotoxins by sourdoughs, proved to be very similar, so it is difficult to point of the sourdough which decompose mycotoxins in the best way. The highest average degradation of mycotoxins was detected in the dough with sourdough, which also obtained the worst results in degradation of nivalenol. *Lactobacillus fermentum* received an average degradation rate of 48%. This result proved to be the best among all obtained during degradation of mycotoxins. These bacteria were also obtained the best result in the reduction of deoxynivalenol, reducing it by 70.7%.

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Effect of DON and DOM-1 on cell viability of IPEC-1 and IPEC-J2 cells

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The mycotoxin deoxynivalenol (DON), often found in cereals for animal nutrition, leads to vomiting, diarrhoea and losses in performance in pigs. Some microbes in intestine have been described to degrade DON. The strain *Eubacterium* BBSH 797, which was isolated out of rumen fluid is able to reduce DON into its non-toxic product deepoxy-deoxynivalenol (DOM-1) (1). As pigs are very sensitive to DON, an *in vitro* screening assay with polarised intestinal porcine epithelial cells (IPEC-1 and IPEC-J2) was established to compare the cytotoxic effects of DON and DOM-1. In the developed test system, cells were seeded in 96-well plates in a density of 1.5 x 10^4 cells/well (IPEC-1) and 1.2 x 10^4 cells/well (IPEC-J2). After incubating cells for 3 days (39 °C, 5% CO_2), DON and DOM-1 were added in a range of 31.25-1,000 ng/mL. Cell viability was measured with a WST-1 assay after further 24 hours incubation.
DON at 250 ng/ml reduced cell viability by 25% of IPEC-1 and IPEC-J2 cells. Both cell lines showed a comparable sensitiveness to DON, whereas IPEC-J2 cells were slightly more sensitive to 31.25-125 ng/ml. DOM-1 was not cytotoxic. Viability of both cell lines was ~98% at 1000 ng/ml DOM-1 (Figure 1). It was shown that the established assay is sensitive to DON but not to its deepoxidized metabolite DOM-1.

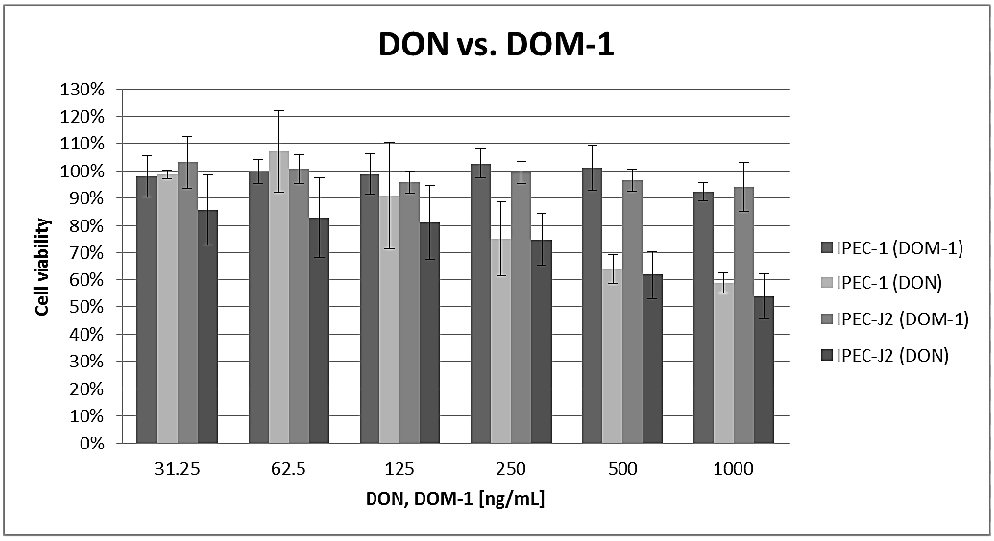

Figure 6 Cell viability [%] of IPEC-1 and IPEC-J2 cells after 24 hours incubation with DON/DOM-1 determined by the WST-1 assay; data represent mean +/- standard deviation of three replicates in two independent experiments.

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Characterization of the fumonisin B_1 (FB_1) decomposing bacterium *Sphingopyxis* sp. MTA144

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Fumonisins are mainly associated with maize based foods and feeds and have toxic and cancer promoting effects on animals and humans. Previously, we isolated the fumonisin degrading alphaproteobacterium *Sphingopyxis* sp. MTA144, and investigated the genes and enzymes enabling catabolism of fumonisins (Heinl et al., 2010). Our present goal was to study the regulation and physiological significance of the *fum* genes in strain MTA144.
We cultivated strain MTA144, harvested and resuspended the biomass in buffer with or without FB_1 for two hours, washed and homogenised the cells, cleared the lysates by centrifugation, and incubated them with 10 mg/l FB_1. The induced lysate catalysed much faster hydrolysis of the mycotoxin. We concluded that the *fum* genes are not constitutively expressed, but inducible. The *fum* gene cluster harbours two genes for transcriptional regulators, which may be responsible for the observed regulation of gene expression. FB_1 degradation experiments with biomass of strain MTA144 showed that FB_1 was not only catabolised in mineral medium, but also in complex growth medium, indicating that there is little or no catabolite repression in the gene regulation. Moreover, we found that the presence of up to 100 mg/ml FB_1 in the medium promoted growth of *Sphingopyxis* sp. MTA144, but had no effect on related strains which do not catabolise fumonisins. This result indicates that *Sphingopyxis* sp. MTA144 degrades FB_1 for energy gain or as a carbon source rather than for self-protecting detoxification.

Reference

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Study on clays as mycotoxin binding agents

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Silicate minerals are widely used and a lot of effort has been put into characterisation of these minerals and studying the effect of different treatments on efficacy. Determination of the right treatment to maximise efficacy is not straightforward because of the complexity of the minerals. It is therefore very important to characterise (un)treated silicate minerals by means of different techniques in order to get as much information as possible on the specific properties of the minerals. This will lead to better prediction of changes in efficacy.

A lot of information is available on the activation of silicates, more particular clays, in order to optimize their efficacy. Common strategies are chemical activation using acid or base; physical activation using grinding, milling or sieving; thermal activation using heat or steam and organic activation using ionic surfactants like sodium dodecyl sulphate. Applying activation techniques onto clays can drastically change their characteristics and hence their efficacy. For instance grinding of clays can increase their specific surface areas and thereby their ability to adsorb compounds. However grinding for long period or using high grinding forces can result in complete structural loss of the clay with collapse of the pores. Consequently, binding of compounds drastically decreases.

How can all the above be related to mycotoxin binding strategies? In order to avoid mycotoxicosis, several strategies have been investigated which can be divided into pre- and postharvest technologies and into biological, chemical and physical methods. The physical methods are focused on the removal of mycotoxins by different adsorbents added to mycotoxin-contaminated diets. Recent studies have investigated clay's adsorption capacities in various applications including the removal of mycotoxins. Clays are silicate minerals which differ principally in the present dominant elements such as sodium, calcium, potassium, magnesium, iron and aluminium. Almost none of natural clays possess broad spectrum mycotoxin binding properties and bind aflatoxins only. However, clays may be processed using some form of treatment to improve their binding capacity. In fact the greatest impact on the mycotoxin binding properties is linked to the type of processing. Using activation strategies and the described techniques, specific clays can be designed for optimal/specific binding efficacy towards mycotoxins.

The work presents the importance of the origin and processing for the mycotoxin binding performance of clays.

The effect of EU maximum levels of 3 mycotoxins and TOXO-XL on tight junction protein gene expression in weaned piglets

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An optimally functioning gut barrier is essential for animal health and performance. Poor gut integrity can lead to an increased risk of infection and malabsorption of nutrients. Several studies show that mycotoxins, for example DON and OTA, have a negative impact on gut barrier function and increase permeability by removing specific tight junction proteins from the tight junctions (e.g. McLaughlin et al. 2004, Pinton et al. 2009). Therefore it is important to focus on this mode of action through which Fusarium toxins exert their negative effects on animal health and performance.

In this study, a trial was conducted to evaluate the protective effect of the feed additive TOXO-XL on animals receiving a feed contaminated with multiple mycotoxins, including DON and OTA, with special focus on counteracting mycotoxin induced tight junction damage.

The experiment was set up together with the University of Utrecht and performed with 200 weaned piglets at the Nutreco Swine Research Centre. The animals were randomly distributed in 40 pens located in 2 rooms with 5 piglets per pen. The experiment included 4 treatments. Two groups were fed a diet low in mycotoxins, with or without TOXO-XL and the other two groups a diet with EU maximum levels for DON, OTA and ZEA with or without TOXO-XL. At day 39 ten animals per treatment were sacrificed and duodenum and colon samples were taken to measure tight junction protein gene expression at the University of Utrecht.

The feed containing EU maximum levels of DON, OTA and ZEA led to an increase in gene expression of the tight junction proteins claudin-7 and occludin in the duodenum. The addition of TOXO-XL reduced the gene expression back to normal values. The gene expression of ZO-1 in the duodenum did not significantly change. In the colon no significant differences were found in gene expression of any of the studied proteins. The results suggest that TOXO-XL reverses the effect caused by the mycotoxins.

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Counteracting the negative effects of mycotoxins in turkeys by supplementation of a mycotoxin inactivator into the diets

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The negative effects of mycotoxins in poultry have been widely published and the mode of actions varies between them. Due to the variety of mycotoxin contamination in the diet, the negative effects can differ strongly. This requires a multi-factorial approach to counteract the impact of these mycotoxins. The aim of present study was to evaluate the efficacy of the mycotoxin inactivator (UNIKE® Plus) in counteracting the negative effects caused by mycotoxins contamination on growth and physiological parameters in turkeys.

Material and Methods

In this study 216 hybrid large white tom turkey poults were raised to 20 weeks of age in 12 pens with each 12 poults per pen.
There were 3 treatment groups:
- Negative control: mycotoxin free diets, no inclusion of mycotoxin inactivator
- Positive control: mycotoxin contamination
- Treatment: mycotoxin contamination + mycotoxin inactivator (1,5 kg/ton).

Diets with naturally Deoxynivalenol (0,6 – 5 ppm) and Aflatoxin (26-164 ppb) contamination were fed to the turkeys during the trial period. The efficacy of the mycotoxin inactivator was evaluated by means of the performance data and physiological parameters.

Results

The results of the current trial demonstrate that mycotoxins negatively affected the performance, organ and immune status of turkeys. Body weight and feed consumption were decreased, while the feed conversion ratio was increased. The immune status and organ morphology were also negative affected by the mycotoxins. Inclusion of UNIKE®Plus in the diets is showing a reduction of these negative effects caused by mycotoxins.

Conclusion

Based on the results of the trial it can be concluded that UNIKE®Plus is an efficacious feed additive to protect turkeys against the negative effects of mycotoxins, it improved performance parameters and established a recovery of organ and immune status.

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List of participants

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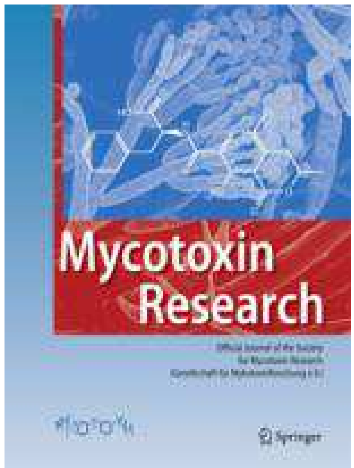

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