Westfälische Wilhelms-Universität Münster

› 31st Mycotoxin-Workshop

Münster, Germany

15th - 17th June, 2009

Conference Abstracts

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Society for Mycotoxin Research

Westfälische Wilhelms-Universität Münster

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Program

Abstracts

Participants

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Scientific and Organizing Committee

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Organization Assistance

Homepage

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Conference Proceedings

Gordon van 't Slot

The 31st Mycotoxin Workshop 2009 would not have been possible without the support of our dedicated staff **Marlies Kleimann**, **Sandra Bieling-Schürhoff** and **Dr. Benedikt Cramer**, as well as the whole **working group** of Prof. Dr. Hans-Ulrich Humpf and many other helping hands at the Institute of Food Chemistry.

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Wednesday, 17th June 2009

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Mycotoxins in food and feed – a sum up of 6 years LC-MS/MS in routine analysis, pros and cons

Scarlett Biselli, Simone Staiger

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The use of LC-MS/MS is more and more the analytical technique of first choice for food and feed analysis. Application of mass spectrometry in combination with modern chromatography leads often to good sensitivity as well as high selectivity. But what about precision, accuracy and reproducibility of these new developed methods? Do these technologies fit in a better way the requirements for food and feed control in terms of Mycotoxin analysis? How is the actual situation with regard to harmonized approaches, what do we know about LC-MS/MS as a reference method?
Within the presentation these questions will be discussed based on examples taken from the application of LC-MS/MS methods in routine analysis for altogether 6 years now. A comparison of pros and cons including the economic aspects will be given.

Analysis of *Fusarium* toxins - Matrix effects and strategies for compensation

A. Trebstein[1,2], U. Lauber[1], H.-U. Humpf[2]

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Fusarium toxins (e.g. T-2 and HT-2 toxin, DON, ZEA and fumonisins) can cause several adverse health effects to animals and humans which is proven by different studies. Therefore, maximum residue levels are planned or have been adopted for relevant food matrices by the European Commission (1). According to the QuEChERS-protocol an HPLC-MS/MS determination method for T-2 and HT-2 toxin, DON and ZEA was developed and tested for checking compliance of foodstuffs with the Commission regulation. For the analysis of fumonisins a dilute-and-shoot HPLC-MS/MS method was tested. Since matrix effects always have to be considered in mass spectrometric analyses, compensation via standard addition as well as via isotopically labelled internal standards was investigated. Fig.1 shows exemplary results of these experiments. In summary, both protocols are useful for screening and under certain conditions even as reliable determination methods, which fulfill all performance criteria stated by the European Commission (2).

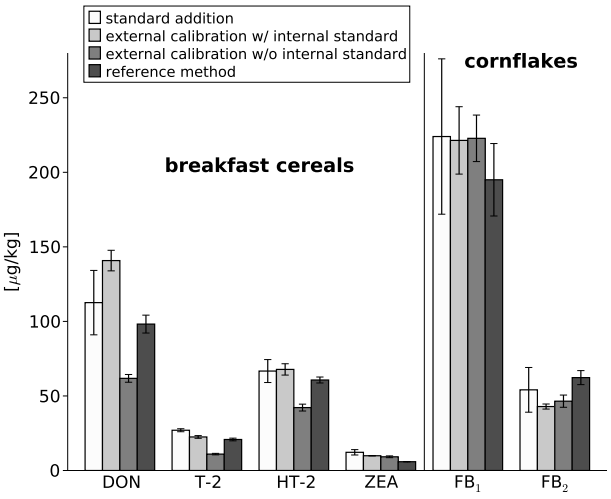

Fig.1: Concentration of toxin ± SD [µg/kg] dependent on the calibration type (n=3). The reference method for DON, T-2, HT-2 and ZEA included a Bond Elut Mycotoxin cleanup and HPLC-MS/MS detection with internal standard correction. The reference method for fumonisins was based on an IAS-cleanup and HPLC-FL detection.

References:

(1) Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. Off. J. Eur. Union 2006, L364, 5-24.
(2) Commission Regulation (EC) No 401/2006 of 23 February 2006 laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs. Off. J. Eur. Union 2006, L70, 12-34.

Multi-mycotoxin analysis using triple stage quadrupole and linear ion trap-Orbitrap

S. Zühlke, D. Herebian, M. Lamshöft, M. Spiteller

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In this study we have compared two different LC-MS platforms for the analysis of mycotoxins in wheat and maize. The first analytical method was based on HPLC-MS/MS technique using a triple quadrupole mass spectrometer (TSQ Quantum Ultra AM). This application analysis was operated in SRM mode with two transitions monitored for each compound. Recoveries obtained for target analytes ranged from 73 to 152% for maize and from 87 to 131% for wheat. The validation results demonstrated high sensitivity, repeatability and linearity of the above mentioned method. Quantitation carried out using a calibration curve constructed using standards including internal standards. The second analytical method was based on microcapillary-HPLC LTQ-Orbitrap. The application of this method in full scan mode is a time-saving method allowing rapid identification and quantitation of mycotoxins at the very low picogram level. In our study, only positive ionisation is applicable to micro-LC LTQ-Orbitrap, resulting in the detection of only 30 out of 32 compounds. Negative Ionization at low solvent flow (4 µl/min at micro-LC) and high water content is problematic due to unstable electrospray.
The sensitivity of both methods applied in absolute injected amounts is in a similar range though it is difficult to compare all single data. As the injected volume is different (micro-LC-Orbitrap 0.5 µl; LC-Quantum 10 µl), the LOD's in µg/kg for the complete method is around the factor 20 better with the triple quadrupole SRM method.
The study demonstrates that the LTQ-Orbitrap also provides a powerful platform for the quantitative analysis of mycotoxins on account of its high sensitivity and selectivity as well as for generating metabolic fingerprints within a few minutes.

List of mycotoxins analysed:
15-Acetyldeoxynivalenol, 3-Acetyldeoxynivalenol, Aflatoxin B1, Aflatoxin B2, Aflatoxin G1, Aflatoxin G2, Altenuene, Alternariol, Alternariol methyl ether, Beauvericin, Citrinin, Deoxynivalenol, Diacetoxyscirpenol, Enniatin B, Ergocornine, Ergotamine, Fumonisin B1, Fumonisin B2, Fusarenon-X, Gibberilic acid, Moniliformin, Monoacetoxyscirpenol, Neosolaniol, Nivalenol, Ochratoxin A, Ochratoxin B, Patulin, T-2 Toxin, HT-2 Toxin, Verrucarin A, Zearalenone, alpha-Zearalenol

Acknowledgement:
The authors thank the Ministry of Innovation, Science, Research and Technology of the State of North Rhine-Westphalia for financial support.

Reference:
Herebian, D.; Zuehlke, S.; Lamshoeft, M.; Spiteller, M., 2009. Multi-mycotoxin analysis in complex biological matrices using LC-ESI/MS: Experimental study using triple stage quadrupole and linear ion trap-Orbitrap. Journal of Separation Science, in press.

Ergot Alkaloid and Ricinoleic Acid Contents in Ergot and their Distribution in Rye Products

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The toxicity of ergot is well known since the mediaeval times and is ascribed to its alkaloids, the most prominent being ergometrine, ergosine, ergotamine, ergocornine, ergocryptine, ergocristine and their corresponding -inine-forms. Nevertheless, there is no legal regulation of the ergot alkaloid content in food and feed at present. The physical determination of the ergot sclerotia in grain ears is often inaccurate and has to be replaced by chemical methods [1].
Besides the alkaloids, ergot contains 35 % fat. The fatty acid spectrum of ergot differs from that of rye by existence of ricinoleic acid and absence of linolenic acid. Whereas the amount of ergot alkaloids in ergot varies considerably (HPLC-FLD: 11.5-236.2 mg/100 g, n=47), the amount of ricinoleic acid determined by gas chromatography is almost constant with 10 g/100 g (8.0-13.3 g/100 g, n=45). This induces the possibility to determine the ergot content via the ricinoleic acid content in processed grain products. With the developed GC-FID- method it is possible to detect an ergot content in rye and rye products up to 0.01 %.

Today the modern milling industry with its good grain cleaning procedures is able to minimize the amount of ergot in the flour. However, there are still small amounts of ergot alkaloids with diverse concentrations in the different milling products like meal and semolina. Our own model milling experiments in collaboration with the MRI Detmold show that the amount of alkaloids correlates with the amount of peel.

Reference:

[1] EFSA 2005, Opinion of the Scientific Panel on Contaminants in Food Chain on a request from the Commission related to ergot as undesirable substance in animal feed, EFSA Journal, 225, 1—27.

Acknowledgement:

This research project is supported by the Arbeitsgemeinschaft industrieller Forschungsvereinigungen e.V. (AiF) through the Forschungskreis der Ernährungsindustrie e.V. (FEI, Project 15280N).
We thank Prof. M. Lindhauer, K. Münzig and K. Wolf for the kind collaboration with the milling experiment at the MRI Detmold (Department of Safety and Quality of Cereals).

Quantitation of alternaria toxins by stable isotope dilution assays

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Stable isotope dilution assays (SIDAs) for the determination of the most important mycotoxins of the black mold *Alternaria*, namely alternariol (AOH) and alternariol monomethyl ether (AME), have been developed. For this purpose, deuterated AOH and AME were synthesized by palladium catalyzed protium-deuterium exchange from the unlabelled toxins. Reaction conditions were chosen in such a manner that the formation of the [$^2H_4$]-isotopologues was favored. The synthesized products were characterized by LC-MS, NMR and UV-spectroscopy. Based on the use of [$^2H_4$]-AOH and [$^2H_4$]-AME as internal standards SIDAs were developed and applied to the determination of AOH and AME in beverages using LC-MS/MS. Method validation revealed low limits of detection (AOH: 0.03 µg/kg; AME: 0.01 µg/kg) and limits of quantitation (AOH: 0.09 µg/kg; AME: 0.03 µg/kg), respectively. Recovery from spiked apple juice was 100.5 ± 3.4 % for AOH (range: 0.1 – 1 µg/kg) and 107.3 ± 1.6 % for AME (range: 0.05 – 0.5 µg/kg). Inter-assay precision (expressed as coefficient of variation, CEV) for AOH was 4.0 % (7.82 ± 0.31 µg/kg; vegetable juice, naturally contaminated) and 4.6 % (1.04 ± 0.05 µg/kg; grape juice, naturally contaminated). For AME, a CEV of 2.3 % (0.79 ± 0.02 µg/kg, vegetable juice, naturally contaminated) was obtained. Analysis of fruit juices showed low contamination with AOH and AME in general. Higher values of both toxins were found in wine and vegetable juices. The values for AOH were higher than for AME in nearly any case. However, the developed SIDA has proven to be optimally suited for further studies on AOH and AME content in food samples to obtain further insight into possible health hazards for the consumer.

Opportunities and drawbacks of LC-MS for investigation of mycotoxins in house dust with and without reference substances

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The population in countries with moderate climates spends more than 80 % of their daily time indoors. About 21 % of all flats in Germany have visible damage due to damp. A variety of moulds can grow e.g. directly visible on wallpapers or covered behind furniture or wainscot. *Aspergillus sp*., *Cladosporium sp*., *Eurotium sp*., *Penicillium sp*. and *Wallemia sp*. are the most prevalent moulds in house dust [1]. Particles as well as semi and low volatile molecules accumulate in house dust. Therefore, house dust is an ideal indicator for indoor contamination with mycotoxins. Furthermore house dust is also a possible way of exposition with toxins. More than 400 mycotoxins or metabolites from moulds are known today, but only a few reference substances are purchasable.
In this study, house dust samples were collected from households with or without visible indoor moulds. The mycotoxins were selected for analysis by a literature search regarding their occurrence in building materials or house dust, toxicological aspects and the availability of standards. Aflatoxin B1, Citrinin, Deoxynivalenol, Diacetoxyscirpenol, Gliotoxin, Ochratoxin A, Roridin A and Sterigmatocystin were picked up and quantified by multiple-reaction-monitoring in the tandem mass spectrometer (LC-MS/MS). Concentration levels for example of Sterigmatocystin are in the lower µg/kg range.
Furthermore many reports are dealing with *Stachybotrys chartarum* in water-damaged dwellings. Chemotype S produces the high cytotoxic macrocylic trichothecenes but no reference substances are commercially available. Therefore the LC-MS offers some opportunities for the characterization of target compounds like Satratoxin G and H. They could be identified by their precursor ion and fragmentation pattern. The results were also similar to literature [2]. The identity of the analytes was confirmed by capillary liquid chromatography coupled to a time of flight mass spectrometer (Cap-LC-TOF-MS). In addition the verification of the precursor ion and fragmentation pattern was also done with a quadropole time of flight mass spectrometer (HPLC-Q-TOF-MS).
The physicochemical properties of the selected mycotoxins are different. Thus, the requirements of the analytical methods were very high, especially in such a complex and heterogeneous matrix like house dust. Selected results for the opportunities and drawbacks of the different determination strategies were presented.

References:

[1] Toepfer, I., Butte, W., 2009. Chemische Indikatoren für Schimmelpilze im Hausstaub. Gefahrstoffe – Reinhaltung der Luft, 69, 92-95.
[2] Gottschalk, C. et al., 2008. Detection of Satratoxin G and H in Indoor Air from a Water-Damaged Building. Mycopathologia, 166, 103-107.

Novel strategies for mycotoxin detection

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Current reference methods for mycotoxin determination are chromatographic techniques with mass spectrometry detection. Multi-methods facilitate simultaneous determination of several mycotoxins in essentially any matrix. The availability of isotopically labelled standards for mycotoxins enables the use of the isotope dilution method, which will remain the most reliable analytical technique in foreseeable future. Because of high costs and a limited throughput of instrumental methods, immunochemical techniques (traditionally ELISA) remain the method of choice for the screening of large numbers of samples. Immunochromatography (Lateral-Flow Immunoassay) is establishing its markets as a faster alternative to ELISA. The need for screening large numbers of commodities for mycotoxin contamination makes up for the fact that Lateral-Flow Immunoassay is semi-quantitative at best. Commercial exploitation of other methods based on antibodies (surface plasmon resonance, electrochemical sensors and fluorescence polarization) has so far been limited.

New options for the improvement of mycotoxin detection technologies emerge on one side from the miniaturization of chromatographic techniques (microfluidics, laboratory-on-chip) and on the other side from the replacement of antibodies by DNA aptamers. These developments can be regarded as technical enhancements of known detection principles rather than radical innovations.

Novel, ground-breaking developments in mycotoxin detection are expected to come from indirect, non-destructive assays, known as remote sensing technologies. The goal is to provide instant results where they are needed for immediate decisions (harvesting machinery, grain escalators) by moving the detection from specialized laboratories to the point of service. The availability of sensors for instant on-site detection of mycotoxin-producing pathogens is one of the prerequisites of precision agriculture. Military technologies of two kinds inspire the development of solutions for these goals. The first are optical technologies borrowed from unmanned surveillance systems, based on processing information-rich images and complex optical data with the use of artificial intelligence. The second and probably even more promising technology is the adaptation of miniaturized, mobile mass spectrometers, similar to those used by early warning systems in the military. Fast enrichment technologies for volatiles, as those used in detectors of traces of explosives extracted by a stream of air from the clothes of passengers at some airports, will aid this development. At the low-cost end, arrays of gas sensors connected to a database of diagnostic signatures might replace mass spectrometers for on-site, non-destructive detection of mycotoxins. Characterization of the relationships between volatile patterns, mycotoxin production and mycotoxin content for different commodities remains the main challenge for research before these technologies are adapted in precision farming and in the screening of agricultural commodities for mycotoxin contamination.

A bioassay approach for determining the effect of cooking on fumonisin toxicity

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Fumonisins are considered potential risk factors for cancer and congenital malformations known as neural tube defects (NTD) in populations that consume maize as a diet staple. Cooking variably reduces fumonisin concentrations. However, fumonisin concentrations and potential toxicity of foods might be underestimated due to formation of novel matrix binding or degradation products (Humpf, H.-U.; Voss, K. A. 2004, Effects of food processing on the chemical structure and toxicity of fumonisin mycotoxins. Mol. Nutr. Food Res., 48, 255-269). Therefore, rodent bioassays incorporating sphingoid base analyses and histologic appearance of target organs as endpoints have been used to determine the effectiveness of selected cooking methods to decrease fumonisin toxicity. 1) Nixtamalization (alkaline cooking) of *F. verticillioides* culture material (CM) reduced its fumonisin B_1 (FB_1) concentrations about 75 percent. Reduction increased to 95 percent when the CM was mixed with ground maize before cooking. When equivalent amounts of uncoooked CM and nixtamalized CM were fed to rats, hepatotoxicity (increased tissue sphinganine and apoptotic lesions) induced by the uncooked CM was significantly more severe than that caused by the nixtamalized CM. In contrast, sphingolipid effects and lesions were absent in rats fed the nixtamalized CM-ground maize mixture. 2) Fumonisin B_1 (FB_1) induces NTD when given intraperitoneally (5 mg/kg (=7 µmol/kg) BW or higher) to LM/Bc mice at the critical time (E7-8.5) for neural tube closure. Using this bioassay, hydrolyzed FB_1 (HFB_1) did not cause NTD at doses up to 20 mg/kg (49 µmol/kg). The 20 mg/kg dose of HFB_1 disrupted maternal sphingolipid metabolism slightly whereas 10 mg/kg HFB_1 (25 µmol/kg) did not. In comparison, a dose of 10 mg/kg (14 µmol/kg) FB_1 markedly disrupted maternal sphingolipid metabolism. 3) Extrusion cooking, which combines high heat and pressure, is a proposed method for reducing fumonisins in maize-based products. It reduced FB_1 concentrations of spiked grits and two batches of *F. verticillioides*-fermented grits from 10 to 28 percent. Addition of glucose to the grits before extrusion increased reductions to 75-85 percent and facilitated FB_1-sugar binding; 52-68 percent of the FB_1 was recovered as the *N*-(deoxy-D-fructos-1-yl) FB_1 binding product. In a three week rat feeding bioassay, extrusion with glucose supplementation reduced the severity of kidney lesions induced by one batch of the fermented grits. The reduction was attributed to the lower FB_1 concentration (1.9 ppm) in the diet prepared from this batch of grits compared to the diets formulated with equivalent amounts of the uncooked (12.7 ppm or higher) or other cooked preparations (3.3 ppm or higher). These bioassays indicate that nixtamalization effectively reduces FB_1 in cooked products and provide evidence that the beneficial effect likely involves interactions between FB_1 and maize matrix components in addition to hydrolysis of the mycotoxin. Rodent bioassays will also be useful in further studies to define the conditions under which other cooking methods, including extrusion with glucose supplementation, will safely reduce fumonisins in maize-based products.

Dietary exposure to T-2 toxin / HT-2 toxin in Germany

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To determine the levels of two major trichothecenes, T-2 toxin and HT-2 toxin (T-2/HT-2), in foods in Germany, a total of 3837 food samples (purchased from retail shops or other food stores as offered to the consumer) were analysed between 2006 and 2008. The analytical methods (ELISA, LC-MS/MS, GC-ECD), which were validated in extensive intercomparison studies, typically enabled detection of T-2/HT-2 in various matrices at 0.1-2.0 µg/kg, at 0.5 µg/kg in most cases. The majority of samples were cereals or cereal-based foods, but other matrices were also screened. In about 77% of the analysed samples, positive results for T2/HT-2 were obtained. The overall median value for T-2/HT-2 was around 1 µg/kg, the 90th percentile 7 µg/kg. Considering both food intake and toxin levels, oats/oats products, breakfast cereals, bread, and pasta were identified as relevant sources. Food intake data were used as generated in the German Ochratoxin A study (1), calculations were similar as in the Fumonisin study (2). Using the 50th or the 90th percentile of food intake and the 50th or the 90th percentile of toxin levels, “best”, “mean”, “bad”, or “worst” case scenarios concerning the exposure to T-2/HT-2 were calculated. In the mean case scenario (median food intake x median toxin level), intake of T-2/HT2 was approximately 10% of the tolerable daily intake (TDI, 0.06 µg/kg body weight and day) in adults, and 10-20% of the TDI in children (4-14 years). In the bad case scenario (median food intake x 90th percentile toxin level), the respective values were 20-30% (adults) and 50-80% (children). Even in the worst case scenario the intake was below the TDI in all age groups. It is concluded that average T-2/HT-2 concentrations in foods in 2006-2008 were sufficiently below critical levels.

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Evaluation of fumonisin intake in gluten-free diet

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Fumonisins are known to occur worldwide as natural contaminants of corn and corn-based products. They are associated with several animal diseases and are a potential threat to human health. Fumonisin biomarkers have been sought in the measurement of levels of the toxin in physiological fluids and tissues. The most common fumonisin biomarkers are those related to the disruption of *de novo* sphingolipid biosynthesis, namely elevated levels of the sphingoid base. In particular, the use of sphinganine to sphingosine ratio was widely described in the literature, although their correlation to fumonisin exposure data seem to be problematic in humans (Shephard et al., 2007). Very recently, the occurrence of free and bound fumonisins in gluten-free products from the Italian market has been described (Dall'Asta et al., 2009). Free fumonisins were found to occur in 90% of the samples: the overall median value was below the EU legal limit for foods for human consumption (800 µg/kg). Anyway, considering the limited diet of people suffering of the celiac disease or allergic to other wheat proteins, the incidence of fumonisin contamination may be envisaged as problematic.
In this study, the collected data about fumonisin occurrence in gluten-free products were matched to consumption data. In particular, a group of celiac people (n = 40) and a group of control (n = 40) have been studied over a week and their food consumption data were recorded by means of a 7-day diary. Moreover, the 24 h urine was collected in the last day of recording and analysed for fumonisin biomarker determination. Concerning fumonisin intake, the results showed a significative difference among coeliac group and control, suggesting a higher exposure risk for the former. On the other hand, the urinary biomarker evaluation did not discriminate among the two groups, probably because of the high individual variability recorded among subjects involved in this study.
A critical presentation of the collected data will be given as well as outlooks and remarks concerning fumonisin occurrence in gluten-free products in Italy.

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Effects of Deoxynivalenol (DON) and its derivative DON-sulfonate (DONS) on porcine peripheral mononuclear cells (PBMC)

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Introduction DON contaminated feed might cause health problems in pigs and efforts for detoxification are undertaken to cope with the problem. Wet preservation of DON contaminated cereals with sodium metabisulphite ($Na_2S_2O_5$, SBS) was shown to reduce the detectable DON concentration through formation of DONS. The aim of the present study was to compare the cytotoxicity of DON and DONS on porcine PBMC as these cells were shown to be susceptible to DON when cultured *in vitro* in a dose-related fashion. In addition, a piglet experiment was performed to test PBMC *ex vivo* after feeding piglets a SBS treated feed. For this purpose, both an uncontaminated control triticale and a DON contaminated triticale were included in the piglet diet either untreated (CON, FUS) or SBS treated (CON-SBS, FUS-SBS) and fed for 28 d starting from weaning.

Methods Procedures for *in vitro* and *ex vivo* proliferation assays are described elsewhere and the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) test was used as cytotoxicity indicator.

Results Increasing DON concentrations resulted in a typical dose-dependent inhibition of PBMC proliferation *in vitro*. The DON concentration where a 50 % inhibition of cell proliferation occurred (IC_{50}) was estimated at 1.1 µM while DONS exerted no inhibitory effect. The concentrations of DON and DONS fed in the piglet experiment amounted to 0.156, 0.084, 2.312 and 0.275 mg DON per kg CON, CON-SBS, FUS and FUS-SBS diet, and to <0.05, <0.05, <0.05 and 1.841 mg/kg diet, respectively, and demonstrated that the preservation treatment related decrease in DON was paralleled by an increase in DONS. The proliferation of PBMC isolated from exposed piglets (*ex vivo* study) was decreased after feeding the SBS treated diets irrespective of triticale source although it appeared that these relationships occurred at a generally lower level after feeding the diets containing the contaminated triticale.

Conclusion The proliferation assays revealed an apparent contradiction in that the *in vitro* tests using PBMC isolated from unexposed pigs were unresponsive to graded levels of DONS whereas the PBMCs isolated from exposed piglets (*ex vivo* assay) seemed to be inhibited to some degree by feeding the SBS treated diets. The reasons for these findings need to be clarified.

Effects of deoxynivalenol (DON) on lipopolysaccharide (LPS) stimulated and unstimulated primary porcine hepatocytes

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Introduction: Deoxynivalenol (DON) exerts toxic effects via protein synthesis inhibition and immunomodulatory actions. Synergistic effects of DON and inflammatory stimuli, as lipopolysaccharides (LPS) were previously demonstrated. As the liver is an organ with a high protein turnover rate as well as an organ strongly involved in systemic inflammatory reactions, it can be considered as a target organ of DON. As pigs react most sensitive to DON exposure, the present investigation aimed to examine the effects of DON on primary porcine hepatocytes, simulated or not stimulated by LPS.

Methods: Hepatocytes were isolated from liver pieces of three healthy castrated male pigs by retrograde perfusion. The resulting cells were cultured in supplemented Williams´ medium E without serum containing increasing concentrations of DON with or without 1µg LPS/ml over a time range of up to 48h. Immunocytochemical staining for porcine macrophages showed that approximately 2% of the cells in culture were Kupffer cells.

Results: The RNA expression of TNF-α peaked at 3h, showing synergistic effects of LPS and high concentrations of DON (Figure 1) whereas the supernatant concentrations of TNF-α did not reflect those synergistic effects observed at RNA level (data not shown). The RNA expression of Il-6 was highest after 6h in LPS stimulated cells irrespective of DON exposure and remained elevated thereafter in cultures not co-exposed to DON or to only low concentrations while it declined in cultures co-exposed to 500 and 2000nM DON. This decline due to co-exposure to high concentrations of DON was paralleled by supernatant concentrations of Il-6 (data not shown). Concentrations of albumin in cell culture supernatants were decreased by LPS and by high concentrations of DON (Figure 2).

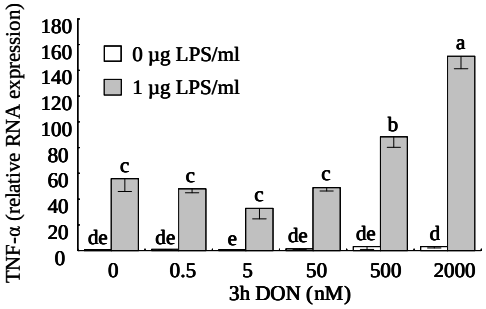

Figure 1 Expression of TNF-α mRNA after 3h incubation with DON in presence or absence of LPS (mean ± SEM)

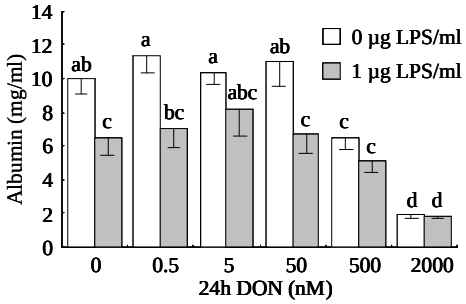

Figure 2 Concentrations of albumin in supernatants after 24h incubation with DON in presence or absence of LPS (mean ± SEM)

Conclusions: The present investigations showed that DON induces immunomodulatory effects in cultures of porcine hepatocytes. Therefore the DON-induced decrease in albumin secretion could be a result of immunomodulation as well as protein synthesis inhibition. The differential effects observed for TNF-α at mRNA and protein level need further clarification.

Lack of effect of T-2 toxin on glucose uptake and electrophysiological parameters in jejunum of broilers

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Introduction: Chicken has been generally considered resistant to the effects of trichothecenes. However, type B trichothecenes at low levels were recently shown to inhibit intestinal glucose absorption in this species. Such data on Type A trichothecenes are not available. Present study was therefore conducted to test the ability of T-2 toxin to affect glucose uptake in jejunal segments from broilers and to investigate if such an effect is a direct result of ion channel modulation.

Materials and Methods: Eight, 28-day old broilers were killed with stunning and bleeding. Jejunum was removed and placed in chilled buffer containing (mM): NaCl, 120 ; $CaCl_2$, 1.2 ; $MgCl_2$, 1.2 ; KCl, 5.5 ; $NaHCO_3$, 25 ; $NaHPO_4$, 2.4 ; NaH_2PO_4, 0.4 ; Mannitol, 20 . After removal of serosa, segments from middle jejunum were mounted in a standard Ussing chamber. The apical and basolateral surfaces of epithelium were bathed with the aforementioned buffer at 37 ºC with continuous carbogen gassing. T-2 toxin was added to apical side at the levels of 0, 0.1, 1, 5, and 10 µg/mL buffer. After 40 and 60 minutes of incubation with the toxin, 7 mM glucose and 200 µM ouabain (a specific blocker of Na^+K^+-ATPase) respectively, were added. Short circuit current (Isc), and transmural potential difference (PD) was measured throughout the experiment. Statistical significance was assessed by using paired-samples *t* test.

Results: None of the T-2 toxin levels affected the electrophysiological parameters of jejunal segments during 40 minutes of incubation. Glucose-induced Isc and PD tended to decrease with increasing concentration of T-2 toxin. Specifically, the glucose induced Isc was 2.9±0.9, 2.1±0.7, 2.9±1.1, 1.8±0.5, and 1.8±0.7 $\mu A/cm^2$ respectively, at 0, 0.1, 1, 5, and 10 μg T-2 toxin/ml. However, these differences were non-significant. T-2 toxin at 5 μg/ml decreased ($p>0.01$) the response from ouabain.

Discussion: A change in electrical parameters of epithelia after application of toxins/drugs is an indication of change in the rate at which these membranes absorb and secrete cations and anions. Since electrophysiological parameters in our study were not affected by T-2 toxin, we can infer that T-2 toxin at the tested levels did not affect movement of ions across jejunal epithelia of chicken during 40 minutes incubation. Previously, 5 µg deoxynivalenol/mL has been shown to affect electrical parameters, epithelial sodium channel, and sodium-dependent glucose cotransporter-1 in jejunal epithelia of chicken (Awad et al., 2005). No such effects were however noted in this study for T-2 toxin even at 10 µg/mL levels.

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Does the exposure of pregnant sows to deoxynivalenol (DON) affect fetal organs? A pathomorphological and immunohistochemical study

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Deoxynivalenol (DON) is a trichothecene mycotoxin that commonly contaminates cereal-based foods and feeds worldwide. It has adverse effects on animals, humans and plants with two important modes of action: (i) inhibition of protein synthesis; (ii) immunomodulatory influence by ribosomal binding and rapid activation of mitogen-activated protein kinases (MAPKs). DON mainly affects tissues and cell populations with high protein turnover such as the liver, intestinal tract as well as leukocytes. Only little information is available about histopathological changings in swine fetuses as only recent studies have proved that DON passes through the porcine placental barrier to a significant extent.
As shown previously solely conventional pathohistological examinations reveal no obvious effects of diaplacental DON intoxication in 70 day old swine fetuses.
In consequence, the objective of this study is to investigate the effects of orally administered DON on immunohistochemical and morphometrical parameters of the immune system, liver and intestinal tract of swine fetuses at day 70 of gestation.
For this purpose 5 sows were fed a control diet (0.15 mg DON and 0.0035 mg zearalenone (ZON)/kg diet) and 7 sows a contaminated diet (4.42 mg DON and 0.0048 ZON/kg diet) between day 35 and day 70 of gestation. On day 70 the fetuses were delivered by caesarean section. Tissue samples of 3 fetuses from each sow were collected, fixed in 4% buffered formalin and processed routinely.
Relating to the modes of action of DON the following antibodies were used in liver, spleen, lymph nodes, thymus, gut and bone marrow to compare control- and treated animals: (i) CD3 and CD79a for the differentiation of lymphocytes in T- and B-lymphocytes; (ii) MAC 387 (myeloid/histiocyte antigen) for the detection of macrophages; (iii) Ki-67 for the detection of cell proliferation; (iv) p-p-53- and p-p-38 MAPK as well as caspase-3 and caspase-8 for the detection of apoptosis (DON induced p38 activation via phosphorylation provokes p53 activation with a following caspase-chain-reaction); (v) TNF-alpha (tumor-necrosis-factor-alpha), is a cytokine for the regulation of immune cells, induction of apoptotic cell death as well as induction of inflammation.
The immunohistochemical findings will be compared between groups and discussed.

Bioavailability of the Fusarium toxin deoxynivalenol (DON) from contaminated wheat straw and chaff in comparison with its grain

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Introduction: Among farm animals, pigs are known to respond most sensitive to DON contaminated feed. The most important source of DON exposure is cereal grain mixed into the diet. However, it is possible that pigs are additionally exposed via straw used as bedding material or as feedstuff. DON from fibre rich plant materials is frequently thought to be bound to plant cell wall components and could consequently be available only to a limited extend for the monogastric pig. Thus, the aim of the present experiment was to scrutinize the bioavailability of DON from straw and chaff in comparison to grain.

Methods: Thirteen castrated male pigs (43.8 kg ± 8.3 kg live weight (LW)) were housed individually in balance cages and equipped with a permanent vein catheter to facilitate the frequent blood collection over a period of 24h. On the day of the kinetic study the diet was substituted by 10-15 % of grain (n=5), straw (n=5) or chaff (n=3) harvested from Fusarium culmorum inoculated wheat. Blood samples were collected 30 minutes before feeding and 0, 10, 30, 45, 60, 90 minutes, 2, 3, 4, 6, 9, 12, 24 hours after feeding. DON in feed and plasma was analysed by HPLC after clean up with IAC (immunoaffinity column). Plasma data of all pigs were fitted to the Bateman function and the bioavailability (F) was estimated as the ratio between dose-corrected areas under curve (AUC) of the tested pigs and pigs that received a single DON dose intravenously.

Results: The DON doses varied between 21.8 µg/kg LW and 37 µg/kg LW because of the different acceptance of the pigs to the straw and chaff and due to the different dietary DON-concentrations. Hence the maximum value of the single functions and also the AUC differ between groups and individual pigs. DON from grain was nearly completely bioavailable (97 percent). The bioavailability of DON from straw was significantly higher (130 percent) than that from grain with a similar trend for chaff (117 percent).

Conclusions: The higher bioavailability of DON from straw compared to grain might suggest that DON from this fibre rich material is available for the animal, but not detectable by routine DON analysis. Thus, there is a need to proof these methods for straw and perhaps also for other feed with high fibre concentrations.

Catechol formation of zearalenone, a novel pathway with toxicological implications

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Introduction: Zearalenone (ZEN) is a common contaminant of all major cereal grains worldwide. Until recently, only reductive metabolites of ZEN were known. Following the disclosure of several monohydroxylated metabolites in incubations of ZEN with NADPH-fortified rat liver microsomes [1], we have now identified the major oxidative metabolites formed by hepatic microsomes from human liver [2] as catechols of ZEN and its reductive metabolite zearalenol (ZEL). The catechols are readily autoxidized to the respective quinones and form covalent adducts with N-acetylcysteine, implying that the catechol pathway of ZEN may lead to electrophilic intermediates.

Materials and Methods: Incubations of ZEN with human hepatic microsomes and rat liver slices were conducted as described [2]. The catechols of ZEN were chemically synthesized from ZEN through nitration to two isomeric mononitro-ZENs, followed by reduction to the respective amines and subsequent oxidation to quinone imines, hydrolysis to quinones and their reduction. The structures of the synthetic compounds were established by ^{1}H- and ^{13}C-NMR spectroscopy.

Results: Four major and two minor monohydroxylated metabolites were detected by LC-MS/MS and GC-MS analysis in incubations of ZEN with human liver microsomes. Using ZEN specifically labelled at the aromatic ring with two deuterium atoms [3] and the synthetic catechol standards, the four major mono-hydroxylated ZEN metabolites were identified as two catechols each of ZEN and ZEL. In support of this structure assignment, all four major oxidative metabolites, but not the two minor ones, were substrates of the enzyme catechol-O-methyl-transferase and gave rise to monomethylated catechols. The methyl ether of one of the aromatic hydroxy-ZENs was also obtained when ZEN was incubated with precision-cut rat liver slices. Incubation of the catechol metabolites of ZEN with N-acetylcysteine in the presence of oxygen lead to the formation of mercapturic acids as identified by LC-MS/MS.

Conclusion: ZEN and its reductive metabolite ZEL undergo aromatic hydroxylation *in vitro*, and the resulting catechols and quinones exhibit electrophilic reactivity. Further investigations are needed to demonstrate this novel pathway *in vivo* and to assess its toxicological significance.

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T-2 toxin and its metabolites: cytotoxicity and metabolism using human cells in primary culture

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T-2 toxin belongs to the large group of trichothecene mycotoxins synthesized by various *Fusarium* molds which can infect raw agriculture materials. Among the trichothecenes, T-2 toxin is one of the most potent mycotoxins and poses a potential health risk in human nutrition. Several acute and chronic toxic effects were observed in humans after consumption of contaminated food. The aim of this work was to determine the effects of T-2 toxin and of several of its metabolites, namely HT-2 toxin, neosolaniol, T-2-triol and T-2 tetraol, on two human cells in primary culture: human renal proximal tubule epithelial cells (RPTEC) and normal human lung fibroblasts (NHLF). Concerning the cytotoxicity of T-2 toxin and its metabolites, different studies were performed with animal cells and cell lines but there are only limited data about cytotoxic effects in human cells available. The use of human cells in primary culture gives a good completion of the already known data because these might be limited due to the disadvantages of cell lines (e.g. immortalization, tumor derivation, longtime cultivation). In order to study the cytotoxicity and mode of cell death, the parameters cell viability, caspase-3-activity and LDH-release were measured after exposure to T-2 toxin and several of its metabolites. With IC_{50} values of 0.2 and 0.5 µM T-2 toxin showed the strongest cytotoxic effect in both cells with triggering apoptosis as kind of cell death starting at a concentration of 100 nM. The metabolites, HT-2 toxin and neosolaniol revealed weaker cytotoxic effects (IC_{50}: 0.7-3.0 µM) and induced apoptosis at higher concentrations (> 1µM). The other metabolites were less cytotoxic (IC_{50}: 8.3 - 25.1 µM) and did not activate caspase-3 up to a concentration of 10 µM. In addition to the analysis of cytotoxic effects, we also studied the metabolism of T-2 toxin in these cells in primary culture. Using LC-ESI-MS/MS we could demonstrate that both cells are able to transform T-2 toxin into HT-2 toxin. Further metabolic activity could only be observed in renal proximal tubule (RPTEC) cells by forming neosolaniol as a second metabolite.

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Action of ochratoxin A on human primary cells

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Ochratoxin A (OTA) is a mycotoxin produced by several fungi growing on food source material. Consumption of OTA is almost unavoidable. The consumption leads to low but detectable amounts of OTA in human blood. The main target of OTA is the kidney. To study the effect and the toxicity of OTA, so far mainly cell lines of different origin have been used which derive mostly from the proximal tubule. To be as close as possible to the situation in humans with respect to the experimental setup, we studied the effect of OTA in human proximal tubule cells (RPTEC) and mesangial cells in primary culture using OTA in the nanomolar concentration range and exposure times up to 14 days. Apoptotic and necrotic cell death, collagen I, III, IV and fibronectin secretion as well as NF-κB activation were studied. In mesangial cells, protein content decreased only when unusual high OTA concentrations were used (1 µM). By contrast, an increase of caspase-3 activity or LDH release was observed after five days already at 10 nM OTA. A decrease of collagen I secretion was accompanied by a virtually unchanged collagen III and fibronectin secretion. Collagen IV secretion was slightly increased at low OTA concentrations (0.3 to 10 nM).
In RPTEC cells, protein content increased when OTA concentrations between 0.3 and 100 nM were used. LDH release indicating necrotic cell death was reduced after 14 days exposure and caspase-3 activity was nearly unchanged at this time point. Cell hypertrophy developed after 14 days exposure to low OTA concentrations. Collagen III and fibronectin secretion was increased indicating a change of extracellular matrix composition. We conclude that OTA has only a minor effect on human mesangial cells in primary culture whereas in proximal tubule cells prolonged OTA exposure leads to cell hypertrophy and to alterations in cell matrix composition. Therefore, the results presented here give good reasons to re-asses the risk of OTA consumption leading to low blood concentrations which have so far been considered harmless.

In vitro studies on the cytotoxicity of selected combinations of mycotoxins

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Contamination of foods and feedstuffs with mycotoxins is a worldwide problem, and ingestion of such products can adversely affect animal and/or human health. Moreover, various fungi can produce more than one toxin, and therefore often several mycotoxins are found as co-contaminations in grains and other crops. Up to now only limited data concerning the effects of combinations of different mycotoxins are published in the literature. In this *in vitro* study, the cytotoxic effects of combinations of ochratoxin A (OTA) and citrinin (CIT), OTA and deoxynivalenol (DON), and enniatin B (EnnB) and DON were determined in V79 cells with the neutral red uptake assay. The basis for selecting these mycotoxin pairs was their co-occurrence either reported in the literature or detected recently in an analysis of wheat and maize from Germany [1]. Moreover, these toxins showed a wide range of cytotoxic potency in V79 cells, with DON being more potent than EnnB and OTA, and CIT as the least potent one [2].
For the combination of OTA and CIT an additive cytotoxic effect was observed over a range of tested concentrations. For the combination of OTA and DON the combined cytotoxic effect was additive at most, and several concentrations showed a less-than additive effect. Another interesting observation was made for mixtures of EnnB and DON that exhibited lower cytotoxicity compared to that of DON alone. The antagonistic effect was significant at EnnB concentrations above 1 µM combined with a wide range of DON concentrations. This was also observed in cultures of human urothelial 5637 cells.
In summary, the combined effects of three relevant mycotoxin mixtures were not stronger than additive when tested for *in vitro* cytotoxicity. Combinations of mycotoxins at levels where only one component exerts cytotoxicity showed no increase in effect. For mixtures of EnnB and DON an interesting antagonistic effect was observed that has to be further investigated in more detail.

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How does VeA effect secondary metabolism in Fusarium fujikuroi?

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Fusarium fujikuroi is known as a rice pathogen that causes hyper elongation of stalks and leaves due to the production of gibberellic acids (GAs). Besides GAs, *F. fujikuroi* may also synthesize other toxins like fumonisins, fusarin C and bikaverin. Although the clustered genes responsible for synthesis of these secondary metabolites are well characterized, our understanding of their regulatory mechanisms is incomplete.
In this study, we identified and characterized the *F. fujikuroi veA*-gene and examined its role as a global regulator of secondary metabolism. Comparison of wild type and *veA*-deletion strains in pathogenicity assays revealed no hyper elongation of rice infected with the deletion mutant, suggesting a down-regulation of GA genes. To identify more genes influenced by VeA in *F. fujikuroi*, we examined changes in gene expression of wild type and *veA*-knock-out strains by use of a *F. verticillioides* oligo microarray. We found that among the down-regulated genes are those involved in sexual development as well as GA and fumonisin synthesis. Up-regulated genes included those involved in bikaverin synthesis. No changes in expression were observed for genes involved in fusarin C production. Northern blot analysis confirmed the microarray data. Examination of *veA*-regulated genes led to the identification of a putative ortholog of *laeA*, a critical regulator of secondary metabolism in *Aspergillus*. The impact of this gene on *Fusarium* secondary metabolism is currently under investigation.
Our results further prove the cross-species use of the *F. verticillioides* microarray to elucidate the diverse effects VeA has on secondary metabolism in *F. fujikuroi*.

Fumonisins and other compounds from *Aspergillus niger*. How can an organism be both industrial workhorse and food safety problem

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The black Aspergilli are some of the most important mycotoxigenic food and feed contaminants, due to their fast growth, pH tolerance and high abundance in many environments.
A. niger is the most important industrial filamentous fungal species used in biotechnology and has been used for citric acid and extracellular enzyme production, biotransformations, bioremediation, and as a nutritious source of single cell protein. Because of its importance three strains of this species have already been full genome sequenced: ATCC 1015, NRRL 3 and CBS 513.88. Both ATCC 1015 and CBS 513.88 have a putative fumonisin gene cluster quite similar to that of a known fumonisin producer *Fusarium verticillioides*, and actual production has recently been reported from several industrial strains. This poses safety problems for the biotech industry since *A. niger* has been given GRAS (generally regarded as safe) status in numerous industrial processes.
Until recently, the main problematic compound has been found to be ochratoxin A, produced in variable amounts within black *Aspergilli*. Yet, no direct correlation between the production levels of ochratoxin A and the damage caused by the fungi was found, although *A. carbonarius* is considered to be the main source in wine and raisins. With the recent findings of fumonisin production from *A. niger*, this necessitates that quite a number of new matrices needs to be screened for fumonisins.

A study of 14 *A. niger* strains and 25 assumed fumonisin producing *Fusarium* species showed that 12 out of 14 *A. niger* strains produced fumonisin B_2 (FB_2). Five *A. niger* and five *Fusarium* spp. were further incubated at six different temperatures from 15-42 ˚C on Czapek Yeast Agar +5% salt or Potato Dextrose Agar. *A. niger* had the highest production of FB_2 at 25-30 ˚C whereas *Fusarium* spp. had the maximal production of FB_1 and FB_2 at 20-25˚C. Addition of 2.5-5% NaCl, or 10-20 % sucrose increased the FB_2 production of *A. niger*, whereas addition of glycerol reduced FB_2 production. All three water activity lowering solutes reduced the fumonisin production of the Fusaria, whereas we observed a large variation between the *A. niger* strains. The results shows that that the regulation of fumonisin production is very different in *A. niger* and *Fusarium* spp., and that stored foods and feeds, often supporting actively growing and sporulating *A. niger* and often containing sugar or salt as preservatives, can potentially be good substrates for fumonisin B_2 production by *A. niger*.

We are subsequently investigating the possibility of a production of fumonisin by *A. niger* in grapes and raisins. Here we have isolated 66 *A. niger*, 4 *A. tubingensis* and 16 *A. acidus* from raisins, 77% of these *A. niger* strains produced fumonisins, neither the *A. tubingensis* nor *A. acidus* produced fumonisins. We found that *A. niger* was able to produce fumonisins B_2 and B_4 on both raisins and grapes in the ppm range, and currently we are screening wine and raisins for these toxins.

Analysis of Major Proteins of *Aspergillus ochraceus* by 2D Gel Electrophoresis and MALDI-TOF/TOF Mass Spectrometry

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Introduction: Mycotoxins are secondary metabolites produced by fungi that contaminate human food and animal feed. Ochratoxin A (OTA) is one of the important mycotoxin due to its high toxicity. OTA, an isocoumarine compound linked to L-β-phenylalanine, is a potent nephrotoxic fungal secondary metabolite produced by various moulds including *Aspergillus ochraceus* and *Penicillium verrucosum*. The proteome analysis of mycotoxin producing mould *A. ochraceus* would help to gain knowledge about proteins which are involved in metabolism (enzymes, transporters) and responsible for mycotoxin production. To obtain a comprehensive overview about the expression of proteins which are concerned with OTA production a proteomic approach based on 2D Gel electrophoresis and MALDI-TOF/TOF mass spectrometry has been applied.

Materials and Methods: *A. ochraceus* was cultivated in malt extract broth at 30°C with shaking at 200 rpm. Cell disruption was performed by grinding the mycelium with liquid nitrogen. Proteins were extracted using lysis buffer (100 mM Tris-HCl, 50 mM NaCl, 20 mM EDTA, 10% v/v glycerol, 30 mM DTT, 1 mM PMSF, 1% v/v protease inhibitor cocktail; 5ml of lysis buffer per gram of mycelia), precipitated with TCA-acetone and then cleaned. The concentration of extracted protein was estimated by Bradford method. The whole protein extract was separated in a 2D-gel electrophoresis system. Proteins were identified using MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Germany) with the help of standard database search engines (e.g., MASCOT). The proteins with low confidence score from database search engines were *de novo* sequenced and were subjected for Homology search to identify proteins.

Results and Discussion: A total of 22 proteins were identified from *A. ochraceus*. These proteins are reported first time in *A. ochraceus*. Out of 22 proteins 13 proteins were identified by MASCOT search engine and 9 proteins were identified by *de novo* sequencing and MS homology search. Most of identified proteins belong to metabolic pathways. Following identified proteins are involved in energy production: NADH-quinone oxidoreductase, nucleoside diphosphate kinase and glyceraldehyde-3-phosphate dehydrogenase. Oryzin/Alkaline proteinase acts as virulence factor in invasive aspergillosis. Autophagic serine protease Alp2 is concerned with regulation of sexual differentiation and autophagy. HSP60 are ATPases and have affinity for unfolded proteins and involved in osmoadaptation. Nevertheless, the present results provide the first proteomic profile from *A. ochraceus* and will be helpful in triggering factors for OTA production.

Exudate droplets produced by toxigenic strains of *Stachbotrys chartarum*

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The production of liquid droplets is a common feature on the surface of fungal mycelia and known as 'guttation'. However, reference to these exudates on various fungal structures is infrequent, and often these guttation droplets are only casually mentioned although this phenomenon is known since more than 120 years (1). Many penicillia and aspergilli exude drops and for *Penicillium* spp. these droplets were used taxonomically. About the mechanism and significance of droplet formation only less is known. In a previous study we have already shown that droplets from ochratoxin A producing *Penicillium* species contain high amounts of ochratoxin A (2).

Aim of our following up study was to examine whether the phenomenon of guttation is also a feature of *Stachybotrys chartarum* isolates and whether toxic metabolites are excreted into these exudate droplets.
For that purpose a total of 30 *Stachybotrys* spp. strains from our collection were cultured on various media and screened for production of exudate droplets. The strains originated from indoor samples from water damaged buildings (e.g. wall papers, gypseum boards, air filters), feed (hay, straw) and food samples (spices). Twelve isolates were found to be *S. chlorohalonata* and 18 of the isolates were identified as *S. chartarum* by morphological analysis, cytotoxic properties, results of EIA testing for trichothecene mycotoxins and the presence of *tri5*-gene.

Our first findings clearly show that almost all isolates were able to produce exudate droplets. The production is, however, dependend on various factors such as incubation time, culture media and size of the agar plates. Droplets were big enough in some cases to be harvested for further analyses.
A high toxic response was measured when testing the droplets of toxigenic *S. chartarum* strains with a commercial EIA kit for trichothecens (QuantiTox, USA) thus demonstrating that toxic metabolites were excreted into these exudates. Work about the metabolite profiling and to analyse more in detail the composition of the droplet is in progress. The preliminary results of this research project clearly demonstrate that active excretion of liquid droplets is also common in *Stachybotrys* spp.

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Indicators and sensor technology for the identification of mycotoxin producing fungi in the processing of grain

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Within the joint research project "ProSenso.net2" (start 2006) a sensor technology for the identification of mycotoxine producing fungi in the processing of grain is developed. In a joint collaborative effort between the ATB and the University of Potsdam different spectroscopic methods (such as UV/Vis/NIR absorption, fluorescence, and reflectance spectroscopy) for the identification of mycotoxine producing fungi and mycotoxins on grain are tested.

For the project cleaned and gamma-sterilized wheat variety CAPO and rye of different varieties was investigated. As test organisms the field borne fungi of the genera *Fusarium* and *Alternaria* and storage fungi of the genera *Aspergillus* and *Penicillium* were used. The moisture content of the grain varied between 11 % and 28 % (Idler 2007). In the fungi inoculation tests grain with a moisture content of 25 % was applied. The screening of mycotoxins such as deoxynivalenol, zearalenone and ochratoxin A in grain samples was carried out using an ELISA test (r-Biopharm).

Non-invasive and non-destructive spectroscopic methods, such as absorption, fluorescence and reflectance spectroscopy, are powerful tools for the detection of mycotoxins in solution and on the surface of grains and flour. Because of its outstanding sensitivity fluorescence spectroscopy based techniques are especially promising for the in-situ detection of mycotoxins such as ochratoxins, aflatoxins as well as zearalenone (Rasch 2008).

With diffuse reflectance spectroscopy information on i) the moisture content of rye and wheat were obtained, ii) grain contaminated with mould and untreated grain were was distinguished, and iii) wheat inoculated with cultures of *Penicillium verrucosum* or *Fusarium graminearum* were successfully discerned from sterilized grain.
The investigation of grains is especially demanding, because of the large variance in size, shape, colour, density, composition e.g., the content of water, starch, and protein as well as texture. Hence, additional chemometric tools are indispensible in order to eliminate these disturbing factors and to extract the desired bio-chemical information with respect to contamination with fungi and/or mycotoxins.

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Functional testing of candidate genes encoding crop plant UDP-glucosyltransferases inactivating deoxynivalenol

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Formation of deoxynivalenol is a virulence factor of *F. graminearum* on wheat, which is required for fungal spread within the infected ear. A resistance QTL (*Qfhs.ndsu-3BS*, gene *Fhb1*) in wheat cultivars with high type II (spreading) resistance co-localizes with a locus allowing efficient detoxification of DON into DON-3-O-glucoside (Lemmens *et al.*, 2005). Using the cDNA-AFLP method a fragment encoding a candidate UDP-glucosyltransferase (UGT) was identified (Steiner *et al*., 2009). The gene fragment TF108 was highly induced by *Fusarium* infection in CM82036 and a doubled haploid progeny DH1 with two resistance QTLs, but not in the susceptible parent Remus and line DH2 with two susceptible QTL alleles. The *Fusarium* inducibility was also tested with real-time RT-PCR. TF108 was induced 134x in CM82036, and 73x in susceptible Remus, and therefore narrowly failed the usual criterion of a two-fold expression difference. Likewise the inducibilities in the closely related DH lines were 54x in DH1 (R) vs. 36x in DH2 (S). To test the hypothesis that TF108 is part of a gene encoding a glucosyltransferase with the ability to detoxify DON, we used heterologous expression in yeast, as previously demonstrated for an *Arabidopsis* gene, *UGT73C5* (Poppenberger et al., 2003). The cloning of a full length TF108 cDNA by RACE PCR turned out to be extremely difficult, only cDNAs with a few sequence deviations from TF108 could be cloned. Diploid grass genomes contain about 200 UGT genes (≈ 600 in hexaploid wheat!), and the C-terminal cDNA-AFLP fragment is located in the highly conserved UDP-glucose binding part. Therefore also candidate rice UGT genes (from a cluster of 4 tandemly repeated genes) were expressed in yeast. No increased resistance was observed, suggesting that despite the promising inducibility, the TF108 gene is most likely not encoding the relevant glucosyltransferase inactivating DON.

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Mycotoxins in the indoor environment

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Molds are ubiquitous in nature and constitute a vital part of ecological systems. Yet, in living areas fungal infestation is undesired. As sufficient water activity is a key parameter for mold growth [1], both, acute water-damage of building materials (plumbing leakage, flooding, etc.) and moisture accumulation in living areas due to the energy-efficient way of constructing (thermal insulation) and modern way of living (insufficient airing, air conditioning), respectively, generate ecological niches favorable for the colonization of fungal species.
Indoor mold growth may on the one hand lead to destruction of building materials and costly remediation. On the other hand, prevalence of indoor fungal growth was epidemiologically found to be “associated” with illness likelihood in subjects living in affected buildings. While mold-related infectious and immunologic effects can be characterized by clinical parameters, toxic effects caused by exposure to specific fungal metabolites are much more difficult to pin down. Literature is devoid of valid indoor fungal metabolite exposure data, which triggers an intense debate about the actual contribution of indoor mold metabolites to the aetiology of indoor environment-related diseases.
Mass spectrometry based screening for various metabolites of indoor fungi has been applied to molds from indoor environments that have previously been inoculated on culture material or on building materials [2]. Direct analysis of indoor air and dust in damp indoor environment has mainly addressed macrocyclic trichothecenes and sterigmatocystin [3,4] produced by the indoor-relevant species *Stachybotrys chartarum* and *Aspergillus versicolor*, respectively.
This presentation intends to give a comprehensive overview on the pattern of fungal and bacterial metabolites that has been obtained by LC-MS/MS based analysis of building materials and dust taken from damp indoor environment. The results are discussed in connection with taxonomic identification of the related fungal strains as well as their capability to produce mycotoxins in culture media.

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Occurrence of mycotoxins in german grains

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Since 2001 the Max Rubner-Institute MRI (Federal Institute of Nutrition and Food, Department of Safety and Quality of Cereals) has analysed harvest samples of different grains for quality and safety. The samples were taken every year from twelve or thirteen German states according to their crop quantity. Different mycotoxins were analysed as well as other contaminants (e.g. pesticides and heavy metals). In the presentation we will show the changes in the Deoxynivalenol (Tab. 1) and Zearalenone content in German wheat and rye from year to year and the occurrence will be compared for both regulated mycotoxins. The appearance of mycotoxins in order of the pre grown crop will be discussed. For other substances of interest (e.g. T2-/HT2-Toxin or ergot alkaloids) investigations will be illustrated on occurrence, distribution and degradation during cleaning treatment and food processing.

Tab. 1 Comparison of DON contents in 2008 for Germany with previous years

grain	year	number of samples	mean	median	min. – max.	90. percentil
wheat	2001	253	246	69	<10 – 3528	722
	2002	261	239	136	<10 – 3616	563
	2003	457	148	51	<10 – 2692	308
	2004	505	268	109	<10 - 3965	714
	2005	496	80	36	<10 - 4097	180
	2006	471	88	16	<10 - 7543	131
	2007	481	394	163	<10 – 12249	763
	2008	468	70	16	< 5 - 2506	185
rye	2001	189	56	14	<10 - 1057	216
	2002	196	153	39	<10 - 4111	196
	2003	276	33	25	<10 - 495	63
	2004	274	145	35	<10 - 3565	310
	2005	265	66	26	<10 - 1672	166
	2006	263	32	< 10	<10 - 1197	43
	2007	241	88	23	<10 - 1606	183
	2008	276	29	17	< 7 - 467	63

contents in µg/kg air-dried grain

Determination of the *Alternaria* mycotoxin tenuazonic acid in cereals and beer and studies on its degradation in aqueous solution

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Although derivatization techniques often appear obsolete in HPLC-MS/MS analyses, they feature a number of significant advantages. Especially in conjunction with 'problematic' (i.e. small, polar and acidic) compounds like the *Alternaria* mycotoxin tenuazonic acid (TA), derivatization can be a powerful tool in order to improve LC-MS/MS results.

We have developed a novel method for the quantification of TA by HPLC-ESI multistage MS after derivatization with 2,4-dinitrophenylhydrazine (Fig. 1), a reagent commonly encountered in environmental analysis (Vogel *et al.* 2000). Workup protocols for solid cereal matrices as well as beer were established. In this talk, method details are given and results from the analysis of 27 cereal and 43 beer samples obtained from local supermarkets in Germany are presented.

To understand the stability of the analyte in aqueous solution, we have furthermore conducted a 4 month stability study at different pH-levels and temperatures. It was shown, that TA is significantly degraded under acidic conditions typically encountered in food matrices (pH = 3.5). At 25 °C, the TA half-life was 307 days. Raising the temperature to 40 °C lowered this value to 78 days. The primary degradation product could be identified and was quantified throughout the stability study. Detailed results will be presented.

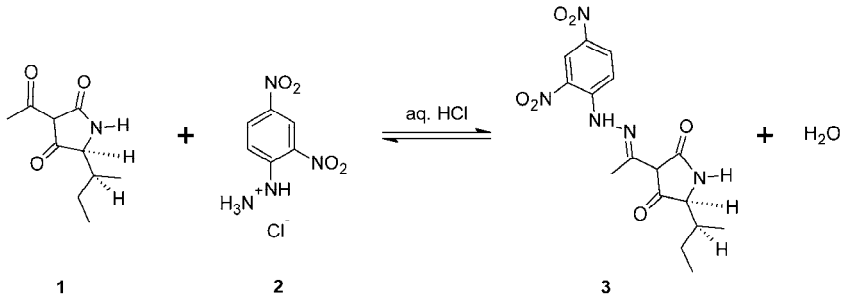

Fig. 1: Derivatization of tenuazonic acid (1) with 2,4-dinitrophenylhydrazine (2)

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The occurrence of mycotoxins in spelt cultivated in organic farms in Poland

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Spelt (*Triticum spelta* L.) is one of the oldest cultivated crops, closely related to common wheat (Suchowilska, Wiwart 2006). Spelt has become an alternative crop in relation to wheat. Spelt is rediscovered in Europe and North America mainly due to the nutritional value (Ruibal-Mendieta et al. 2002).
The occurrence of four mycotoxins was investigated in samples of spelt growing in different organic farms. The kernels of spelt were sampled in the summer of 2008 in organic farms in north-eastern Poland. The Enzyme-Linked ImmunoSorbent Assay (ELISA) method was used to identify aflatoxins, ochratoxin A, toxin T-2 and deoxynivalenol.
Among 9 of the examined samples of winter spelt deoxynivalenol was identified in all samples (up to 0.31 mg/kg), T-2 toxin, aflatoxins and ochratoxin A were not found.

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Nike L. Ruibal-Mendietaa, N., Delacroixb, D., Meurensa, M., 2002. A Comparative Analysis of Free, Bound and Total Lipid Content on Spelt and Winter Wheat Wholemeal. Journal of Cereal Science, 35, 337-342.
Suchowilska, E.; Wiwart, M., 2006. Multivariate analysis of image descriptors of common wheat (*Triticum aestivum*) and spelt (*T. spelta*) grain infected by *Fusarium culmorum*. Int. Agrophysics, 20, 345-351.

Chemistry and biology of selected mycotoxins

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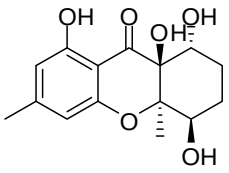
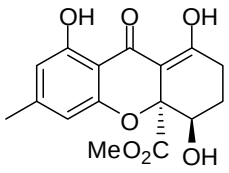
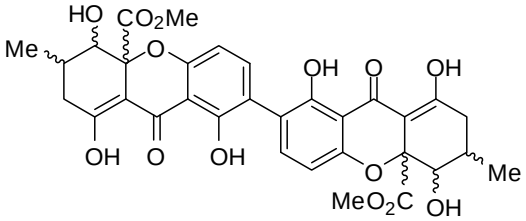
Mycotoxins belong not only to a structural diverse class of compounds, they also display a broad range of biological activities. In a program to use their molecular architecture for the generation of medicinally relevant new entities, the total synthesis of some mycotoxins and the preparation of analogs are targeted.
The tetrahydroxanthone-structure is a common motive for mycotoxins, including diversonol (**1**), blennolide C (**2**) and secalonic acids (**3**). However, only a few total syntheses of natural tetrahydroxanthones have been reported so far. The total synthesis of diversonol, blennolide C will be presented using an oxa-Michael reaction as a key step.



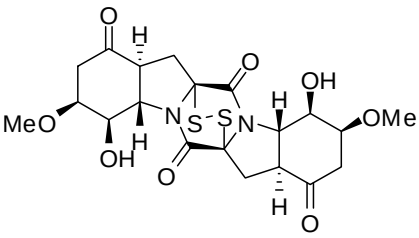
Diversonol (**1**)



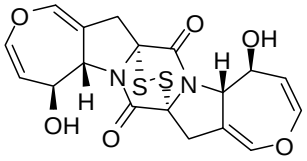
Blennolide C (**2**)



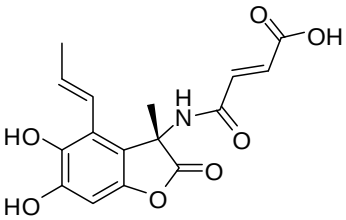
Secalonic acids (**3**)



Rostratin C (**4**)



Aranotin (**5**)



Fumimycin (**6**)

Aside, progress towards the syntheses of the marine mycotoxin rostratin (**4**) and the related aranotin (**5**), both being epithiodiketopiperazines with interesting biological properties, is presented. The bacterial peptide deformylase (PDF) inhibitor fumimycin, another target compound of our group, is a metabolite incorporating an unusual alanine unit linked to a phenyl group at the α-carbon with both lactone and amide moieties which was isolated from cultures of *Aspergillus fumisynnematus*.

References:

Nising, C. F.; Ohnemüller, U. K.; Bräse, S., 2006. The Total Synthesis of the Fungal Metabolite Diversonol. Angew. Chem. Int. Ed., 45, 307-309; Angew. Chem., 118, 313-315.
Gérard, E. M. C.; Bräse, 2008. Modular syntheses of diversonol-type tetrahydroxanthone mycotoxins: blennolide C (epi-hemirugulotrosin A) and analogs.
Chem. Eur. J., 14, 8086-8089.
Bräse, S., Encinas, A.; Gall, J.; Nising, C. F. 2009. The Chemistry and Biology of Mycotoxins. Chem. Rev., 109, in press.

Occurrence of *Fusarium* toxins in potatoes

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Two HPLC-MS/MS methods including 19 *Fusarium* toxins (3,4,15-triacetoxyscirpenol, 4,15-diacetoxyscirpenol, 4-monoacetoxyscirpenol, 15-monoacetoxyscirpenol, scirpentriol, acetyl T-2 toxin, T-2 toxin, HT-2 toxin, T-2 triol, T-2 tetraol, neosolaniol, verrucarol, deacetylverrucarol deoxynivalenol, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, nivalenol, fusarenon X and zearalenone) were established to determine their occurrence and quantity in potatoes and potato based products. Internal standards (^{13}C-diacetoxyscirpenol, ^{13}C-15-monoacetoxyscirpenol, ^{13}C-T-2 toxin, ^{13}C-HT-2 toxin, ^{13}C-deoxynivalenol, and zearalanone) were used to improve the reliability of the method. Limits of quantification were between 0.2 µg/kg and 19.4 µg/kg.

To check the burden with the examined mycotoxins, a total of 206 potato and potato based product samples were bought by retail. 30.1 % of those samples were positive for at least one toxin analysed. 71 % of all positive samples contained more than one toxin. Maximum concentrations in potatoes were 88.8 µg/kg (diacetoxyscirpenol), 253.9 µg/kg (15-monoacetoxyscirpenol), 24.6 µg/kg (T-2 toxin), 55.3 µg/kg (HT-2 toxin), and 9.7 µg/kg (neosolaniol). Scirpentriol and 4-monoacetoxyscirpenol were also traceable.
Several toxins of the scirpentriol group were detected in mashed potatoes. The sample with the highest toxin level contained diacetoxyscirpenol (7.1 µg/kg) and 15-monoacetoxyscirpenol (25.0 µg/kg), as well as scirpentriol and 4-monoacetoxyscirpenol. Deoxynivalenol and zearalenone occurred both in potatoes and in several potato based products such as potato dumplings, mashed potatoes, potato fritter and chips. No toxin content was observed in boiled potatoes, fried potatoes and crisps.

Spread of *Fusarium sulphureum* MRC514 and a *tri5* mutant in 22 potato cultivars: trichothecene production increases virulence

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Fusarium sambucinum causes dry rot of potatoes and produces type A trichothecenes such as diacetoxyscirpenol (DAS) and T-2 toxin. Despite the high toxicity and large amounts of toxins produced in infected tissue, trichothecene productions is generally believed to be irrelevant for virulence, based on the report of Desjardins et al. that a *tri5* gene disruption mutant of their tested strain showed unchanged virulence on the tested potato cultivar. We have reinvestigated this question using a different strain, *Fusarium sulphureum* MRC514 (*F. sulphureum* is a synonym of *F. sambucinum*). This strain originates from an infected potato from Iran and produces large amounts of DAS and monoacetoxyscirpenol in liquid media (Steyn et al., 1978).
A *tri5::HYG*^{R} gene disruption mutant of MRC514 was generated and used in virulence tests on a set of 22 potato cultivars grown and marketed in Austria. The strain showed unchanged growth on plates but lacked DAS production, as expected for a mutant with the first gene of trichothecene biosynthesis inactivated. Potato tubers were surface sterilized and inoculated with cork borer sized fragments from PDA agar plates containing either the wild-type or mutant strain. Disease symptoms were recorded 5, 10, 15 and 20 days post inoculation, and concentrations of DAS and ergosterol (ERG) were measured with a novel LC-MS/MS method after a two step extraction procedure. The *tri5* mutant showed clearly reduced disease symptoms on all cultivars. Accordingly, strongly reduced ergosterol amounts reflecting reduced fungal biomass were measured in the tissue infected by the *tri5* mutant. Large differences between cultivars in ERG and DAS accumulation were also observed between cultivars infected with the wild-type. We conclude that trichothecene production is relevant for virulence of *F. sulphureum* MRC514, and that quantitative differences in the ability to restrict fungal growth and toxin production exist in the tested potato cultivars.

References:

Desjardins, AE.; Hohn, TM.; McCormick, SP., 1992. Effect of gene disruption of trichodiene synthase on virulence of *Gibberella pulicaris*. Mol. Plant Microbe Interact., 5, 214-222.
Steyn, PS.; Vlegaar, R; Rabie, CJ.; Kriek, NPJ.; Harington, JS. 1978. Trichothecene mycotoxins from *Fusarium sulphureum*. Phytochem., 17, 949-951.

Funded by the Austrian Science foundation FWF (SFB F37 and L475-B09).

Fusarium mycotoxins in maize in two vegetation periods differing in environmental conditions

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Maize ear rots caused by *Fusarium* spp. occur worldwide and are a serious threat to human and animal health arising from capability of most *Fusarium* species to produce mycotoxins. Maize kernels infected by *F. verticillioides* and *F. proliferatum* are primarily contaminated with fumonisins. Type B-trichothecenes deoxynivalenol (DON) and nivalenol (NIV), and the estrogenic compound zearalenone (ZON) are also frequent contaminants of maize ears, due to the common presence of *F. graminearum*, *F. culmorum* and *F. cerealis* (syn. *F. crookwellense*). Fumonisin-producing species are the prevalent pathogens associated with maize ear rots in Southern Europe whereas *F. graminearum* predominates in years and regions with frequent rainfall and moderate temperatures (Logrieco et al. 2002, Munkvold 2003). However less is known about the mycotoxin profile associated with *Fusarium* ear rots on maize in Germany. In 2006 and 2007, therefore, a total of 84 maize crops was analysed in order to assess the variability of *Fusarium* infection and mycotoxin contamination. As weather conditions largely varied between years the influence of temperature and rainfall during the growth period on mycotoxin spectrum and concentration could be evaluated. A multi-mycotoxin determination method detecting 32 fungal secondary metabolites, based on a liquid chromatography-mass spectrometry was used. Meteorological data were obtained from weather stations operated by the German meteorological service. Weather stations were located within an average distance of 15 km to the sampled fields. In both years, weather information was retrieved for the period from 1 Mai to 30 October.
Environmental conditions significantly influenced the profile and concentrations of mycotoxins associated with *Fusarium* ear rot on maize in Germany. Fumonisin contaminations were detected more frequently in 2006 than in 2007. DON and its acetylated derivates 3-AcDON and 15-AcDON were commonly observed in both growth periods. Frequency and median concentration of DON and 15-AcDON, respectively, were considerably higher in 2007. ZON contaminations were detected in 23 % of samples in 2006 whereas the number of samples positive for ZON was higher in 2007. Moniliformin, NIV, beauvericin and enniatin B were common contaminants of maize kernels in both vegetation periods. Type A-trichothecenes T-2-toxin, HT-2-toxin and monoacetoxyscirpenol were detected infrequently in both years. Correlations between maximum, minimum and average temperature as well as rainfall, and mycotoxin contaminations were observed in both growth periods.

References:

Logrieco, A.; Mule, G.; Moretti. A.; Bottalico, A., 2002. Toxigenic *Fusarium* species and mycotoxins associated with maize ear rot in Europe. Eur. J. Plant Pathol., 108, 597-609.
Munkvold, G.P., 2003. Epidemiology of *Fusarium* diseases and their mycotoxins in maize ears. Eur. J. Plant Pathol., 109, 705-713.

Abstracts of Posters

Evaluation of extraction solvents for the determination of mycotoxins in sugar beet by LC-ESI-MS/MS

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Sugar beet (*Beta vulgaris* L.) is commonly infected with mycotoxin-producing *Fusarium* species (Bosch et al. 1992: Mycopathologia 119, 167-173). Conventional extraction of mycotoxins from sugar beet with acetonitrile-water is hampered by a high sugar content of the matrix. In the ion source sucrose forms caramel, contaminating the ionization chamber and impairing the performance of mass spectrometer. The purpose of this study was to determine the suitability of ethyl acetate as an alternative extraction solvent for mycotoxins in order to minimize carryover of sugar into the ion source. For comparison, conventional solvents acetonitrile and methanol with and without acidificaton were used. Homogenized sugar beets were spiked with nivalenol (NIV), deoxynivalenol (DON), 3-acetyl-deoxynivalenol (3-ADON), 15-acetyl-deoxynivalenol (15-ADON), fumonisin B1 (FB1), and zearalenone (ZON) and extracted. The residues were dissolved in mobile phase and analyzed by liquid chromatography-electrospray ionization–tandem mass spectrometry (LC-ESI-MS/MS). The recoveries are shown in Figure 1. Adequate extraction of NIV was not achieved with any solvent. Highest recoveries for all trichothecenes and ZON were obtained with ethyl acetate. FB1 extracted poorly into ethyl acetate, best recoveries were obtained with acidified methanol.

Ethyl acetate appears suitable for the extraction of trichothecenes (except NIV) from sugar beet matrix.

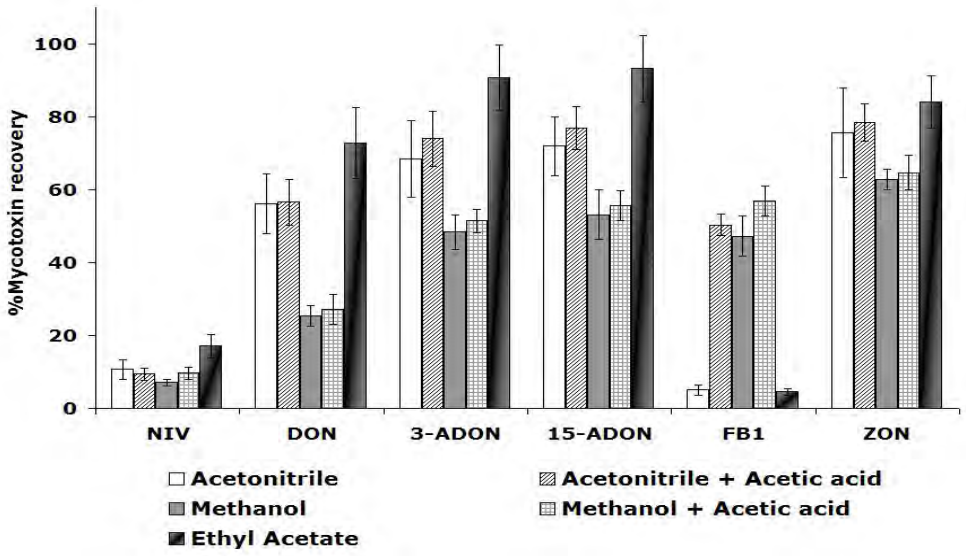

Figure 1: Recovery rates for mycotoxins extracted from sugar beet

Validation of an HPLC-MS/MS based multi-analyte method for the determination of fungal and bacterial metabolites in indoor samples

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Determination of potentially bioactive microbial metabolites (e.g. mycotoxins) in dust and other indoor matrices is inevitable for assessment and the basis to elucidate the actual role of these compounds in the complex health effect pattern of indoor mold exposure [1]. Adequately sensitive and selective analytical strategies to quantitatively determine a broad spectrum of indoor microbial metabolites in exposure-relevant matrices (particularly dust) are not available (with the exception of focused studies [2],[3]). Therefore, the principal technical objective of the present study is to fill this methodological lacuna.
An LC-MS/MS based method for the qualitative and quantitative determination of 172 fungal and bacterial metabolites in indoor samples is developed and validated [4]. Extraction was accomplished in a single step with no further cleanup using acetonitril/water/acetic acid (79:20:1). Method performance parameters such as limits of detection (LOD), linearity, and precision were evaluated by spiking an indoor dust reference material at multiple concentration levels. Extraction efficiencies and matrix effects were determined by spiking blank extracts post extraction.
Indoor dust proved to be an extremely complex matrix, as significant matrix effects were observed for the majority of the analytes. (Other investigated indoor-relevant materials such as mortar and carton-gypsum board were less critical in this regard.) In addition, incomplete extractions were observed for some metabolites. All effects/losses were reproducible at all concentration levels of the spiked reference material, which is reflected by the low values obtained for the coefficients of variation.
The application of the method to real-world indoor samples and the respective culture isolates revealed the presence of more than 30 fungal and bacterial metabolites.

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2. Gottschalk, C., Bauer, J., Meyer, K., 2008. Detection of satratoxin G and H in indoor air from a water damaged building. Mycopathologia 166, 103-7
3. Rintalam, H., Hyvärinen, A., Paulin, L., Nevalainen, A., 2004. Detection of streptomycetes in house dust - comparison of culture and PCR methods. Indoor Air 14, 112-9.
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Development of an UPLC-MS/MS multimycotoxin analysis and application for analyzing the mycotoxin production of pure fungal cultures

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Fungi present in feed or food commodities do not only cause spoilage, but can also result in high amounts of allergenic spores and the production of mycotoxins. Mycotoxins are fungal secondary metabolites with a potential toxic effect on animals and humans. Mostly, food and feed commodities are contaminated with more than one mycotoxin. The presence of a range of mycotoxins implies that synergetic, additive or antagonistic effects can occur after consuming contaminated feed/food. Most of the commercially available detection methods are based on antibody-antigen reaction, which can lead to unwanted cross reactivity and mostly detect only one or just a few mycotoxins. A more recently developed multimycotoxin technique concerns High Pressure Liquid Chromatography coupled to tandem quadrupole mass spectrometry (HPLC-MS/MS). Ultra Pressure Liquid Chromatography (UPLC) is the latest LC method allowing fast analysis, without loss of sensitivity or resolution.

The aim of this study was to develop a multimycotoxin UPLC-MS/MS method for 22 mycotoxins. We started with optimizing the mass spectrometric parameters on the Quattro Ultima Pt that allow the detection of minimum 2 product ions per precursor ion for each mycotoxin standard. Subsequently, the chromatographic conditions were optimized on the Acquity UPLC system with the UPLC BEH C_{18} column (2.1x100 mm, 1.7 µm). For the mobile phases, H_2O + 0.1% formic acid + 1 mM ammonium acetate (solvent A) and ACN + 0.1% formic acid gave the best results. The gradient was initiated with 10% eluent B for 3 min, continued with a linear increase to 87% B in 6 min, held at 100% B for 1.5 min and finally held at 10% B for 1.5 min.
This method was applied for evaluating different solvents for the extraction of 22 mycotoxins from the fungal growth medium Yeast Extract Agar (YES). The best extraction procedure consists of subsequent addition of methanol 100%, dichloromethane 100% and ethylacetate 100%. Possible matrix effects, the repeatability (RSD_r), reproducibility (RSD_R) as well as the limit of detection (LOD) were studied. Afterwards, the extraction procedure was applied for the detection and quantification of mycotoxins produced by several fungal isolates grown on YES.
The results will be presented on the poster.

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Simultaneous detection of all currently EC-regulated mycotoxins in maize and maize products by LC-MS/MS

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The fast and quantitative assessment of a multiple mycotoxin contamination of cereals and maize with mycotoxins of the trichothecene, aflatoxin, and fumonisin group, ochratoxin A (OTA), and zearalenone (ZEA) is an important task for food and feed safety. Especially maize is considered to be highly susceptible to the occurrence of various toxins at the same time.

Therefore, a method for the determination of all currently EC-regulated mycotoxins (Commission Regulation (EC) No 1881/2006) within a single LC-MS/MS-run in maize and maize products was developed. Further analytes of interest like T-2/HT-2 toxin, deoxynivalenol-3-glucoside, mycophenolic acid and the hydrolyzed metabolites of fumonisin B1 were included in the method. For quality assurance of the results, ^{13}C-stable isotope standards were applied for compensation of possibly interfering matrix-effects on deoxynivalenol, T-2/HT-2, fumonisins B1-3, and OTA. The other toxins were quantified using a matrix-assisted calibration.

The LODs for type A trichothecenes ranged between 0.1 µg/kg for T-2 toxin and 2.7 µg/kg for HT-2 toxin, for type B trichothecenes between 1.1 µg/kg for 15-acetyldeoxynivalenol and 2.2 µg/kg for fusarenon-X. For aflatoxins, the LODs varied from 0.02 µg/kg for aflatoxin B2 to 0.2 µg/kg for aflatoxin G2. The LODs of fumonisins were between 0.9 µg/kg for partially hydrolyzed fumonisin B1 and 4.6 µg/kg for fumonisin B1. For OTA and ZEA, the LODs were 0.1 µg/kg and 2.2 µg/kg, respectively. The method was validated for maize meal and semolina, cornflakes, and maize noodles and it fulfilled the requirements of Commission Regulation (EC) 401/2006. Details on further characteristics of the method, the clean-up of the samples, and the recoveries will be presented.

References:

Commission Regulation (EC) No 401/2006 of 23 February 2006 laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs. *Off. J. Eur. Union* 2006, L70, 12-34.
Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. *Off. J. Eur. Union* 2006, L364, 5-24.

Glucuronidation of deoxynivalenol

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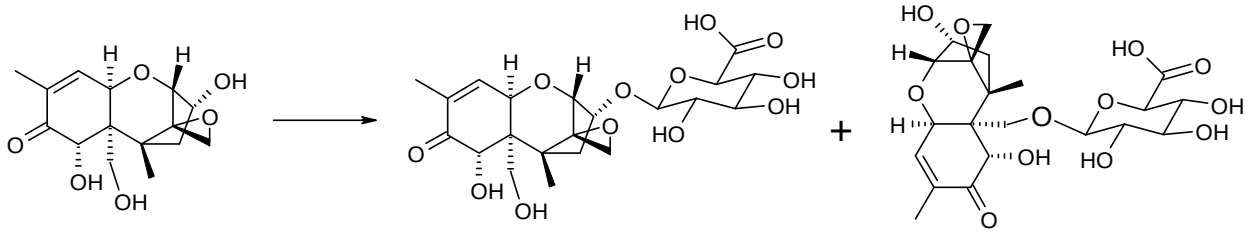
The mycotoxin deoxynivalenol (DON) is a highly toxic and widespread pollutant in grains such as wheat, barley and maize. Although the toxicity of deoxynivalenol is well studied, the effects of its glucuronide metabolites are still unknown. For this reason the glucuronide conjugates should be synthesized to serve as reference compounds for analytical studies.

Due to the high price of the mycotoxin itself, three model compounds were synthesized to mimic the structure elements of DON in the optimization of the glucuronidation reaction. To avoid introduction and removal of protective groups on the target and thus needless loss of starting material we aimed at a highly efficient and regioselective glucuronidation method for DON. A lot of different protocols [1] for glucuronidation reactions are available in literature, for example the Koenigs-Knorr procedure using bromosugars (glucuronic acid derivates) as glucuronyl donors and silver carbonate as activator. These and several other methods (iodosugar or Lewis-acid method) should be evaluated on the model compounds.



Deoxynovalenol **Deoxynivalenol-3-glucuronide** **Deoxynivalenol-15-glucuronide**

Figure 1. glucuronidation reaction

Finally the best working glucuronidation methods should be applied to the mycotoxin in order to synthesize the target structures. Our desired products, which are obtained by glucuronidation of DON in position 3 or 15, respectively, are suggested metabolites of the mycotoxin deoxynivalenol (figure 1).

Reference:

[1] Stachulski A. V.; Jenkins, G. N.; 1998. The Synthesis of O-glucuronides. Natural Product Reports, 15(2), 173-186

Synthesis of 4-acetylzearalenone and zearalenone-2-β-D-glucuronide

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Introduction: The rapid global re-emergence of *Fusarium* head blight disease of wheat and barley in the last decade along with contamination of grains with mycotoxins attributable to the disease have spurred basic research on the fungal causal agent. As a result, *Fusarium graminearum* quickly has become one of the most intensively studied fungal plant pathogens. This work focuses on the *Fusarium* mycotoxin zearalenone, especially the synthesis of its metabolite zearalenone-2-glucuronide, which is suspected to be formed during phase II detoxification of zearalenone in animals (fig. 1).

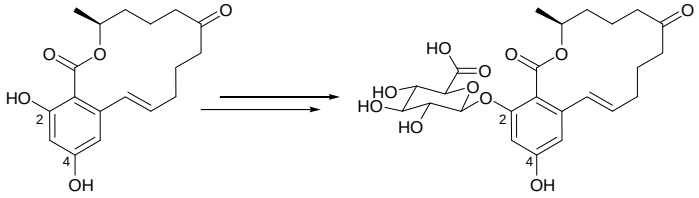

Fig 1 : Synthesis of zearalenone-2-glucuronide

Materials and Methods: Caused by the lower reactivity of the OH-group in position 2 towards glucuronidation, it was necessary to protect position 4. This was realized by synthesis of 4-acetylzearalenone as intermediate before coupling with an appropriate glucuronyl donor. Due to the high price of mycotoxin and the difficult, not well studied glucuronidation reaction, isopropyl 4-acetoxy-2-hydroxybenzoate was synthesized and used as model structure to study and optimize the glucuronidation reaction in position 2. During the glucuronidation reaction this benzoic acid ester reacts as glucuronyl acceptor (G-acceptor) with glucuronyl donors (G-donors), which are basically derivatives of glucuronic acid. In the literature a lot of different glucuronyl donors are known, which are used with different activators (Lewis acids, metal salts, etc.) to synthesize glucuronides [Andrew V. Stachulski, Gareth N. Jenkins, Natural Product Reports, 1998, 15 (2), 173-186]. To screen all these different methods (bromosugars, fluorosugars, trifluoro- or trichloroacetimidates) on our model structure and optimize the procedure for the reaction with 4-acetylzearalenone, these acetyl protected glucuronyl donors were synthesized from D(+)-glucurono-6,3-lactone. Acetyl protection was chosen due to the fact that only one deprotection step is needed in the end of the sequence.

Results: After screening and optimization of all the different methods, we were able to carry out the glucuronidation reaction stereoselectively in position 2 (fig. 1). The deprotection was performed by an optimized protocol we developed during synthesis of zearalenone-4-β,D-glucuronide.

Conclusion: 4-Acetylzearalenone was synthesized and used as intermediate for the synthesis of zearalenone-2-β-D-glucuronide via an optimized glucuronidation protocol.

Synthesis of isotopically labelled alternariol

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Alternaria toxins are mycotoxins produced at the pre-harvest stage of crops as secondary metabolites by species of the genus *Alternaria*. They can be classified in three main structural classes: tetramic acids (e.g. tenuazonic acid), altertoxins (e.g. altertoxin I) and dibenzo-alpha-pyrones (e.g. alternariol). Alternaria toxins have been detected in a wide range of vegetable food and feed (e.g. grain, fruits, vegetables and spices).
Due to the potential risks for the health of humans and livestock as well as possible economic damage caused by alternariol, convenient and reliable methods – such as HPLC-MS/MS - for the detection and analysis in different matrices are needed. The use of HPLC-MS/MS is mostly limited by the effect of matrix compounds on the ionisation of the analyte. These matrix effects can easily be compensated by the use of isotopically labelled internal standards for stable isotope dilution analysis.
A convenient approach for the synthesis of isotopically labelled standard is the introduction of either ^{13}C- or ^{2}H-lables. To ensure a long stability of the labelled standard, ^{2}H-lables should be used in positions which are not prone to deuterium-protium-exchange.
Following a synthesis (Fig. 1) published by Marmor[1] we were able to produce $^{2}H_3$-labelled orcinol by using $^{2}H_3$-acetaldehyde.

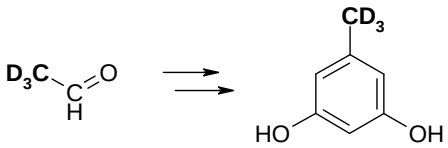

Fig. 1: Synthesis of labelled orcinol

The labelled orcinol was used as a starting material for the synthesis of alternariol (Fig. 2) described by Koch et al.[2].

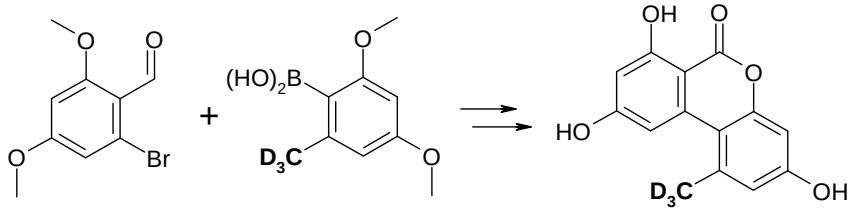

Fig. 2: Synthesis of labelled alternariol

References:

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J. Org. Chem., 37, 2901-2904.
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J. Org. Chem., 70, 3275-3276.

Evaluation of a proficiency test on the determination of ochratoxin A in liquorice

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Within the framework of the German Food Monitoring Programme, proficiency tests are carried out as a measure of external quality control that enable the laboratories to provide data to compare their methods and that might help to recognise systematic deviations. Proficiency tests on the determination of mycotoxins in various matrices are carried out regularly.

The liquorice test material was produced by spiking blank material with ochratoxin A. The material was minced, cryogenically milled and homogenised to slurry with 1 part sample to 1 part water. Homogeneity was tested and confirmed according to the relevant protocols [ISO 13528; Thompson et al. 2006].

Fourteen laboratories submitted results. The participants applied their regular analytical procedures. For evaluation, they were asked to provide information on the methods used. All laboratories used immunoaffinity clean-up and HPLC-fluorescence detection.

The statistical evaluation was performed by applying robust statistics as described in standard DIN 38402. The target value was calculated as 3.10 µg/kg with a relative reproducibility of 16.8 %. Based on target value and standard deviation, z-scores were calculated as a standardised measure of the laboratory performance.

Thirteen laboratories submitted quantitative results within the tolerance limits, whereas one result had to be rated as unsatisfactory due to significant underestimation. In subsequent tests, including various clean-up columns and the analysis of different matrices, this laboratory identified the immunoaffinity columns used during the study as the most probable reason for its underperformance.

References:

DIN ISO 13528: Statistische Verfahren für Eignungsprüfungen durch Ringversuche; 2005
Thompson, M.; Ellison, R.S.; Wood, R., 2006. The International Harmonized Protocol for the Proficiency Testing of Analytical Chemistry Laboratories; Pure Appl. Chem., 78 (1), 145–196
DIN 38402 A 45: Deutsche Einheitsverfahren zur Wasser-, Abwasser- und Schlammuntersuchung, Teil 45: Ringversuche zur externen Qualitätskontrolle von Laboratorien; 2003

Ochratoxin A in liquorice products: International proficiency test

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Ochratoxin A (OTA) is a secondary metabolite of toxigenic moulds, particularly of the genus *Aspergillus ochraceus*. Its occurrence is typical in indigenous, starch-containing cereals, nuts, coffee, and spices, and yet positive findings of this mycotoxin were also established in the liquorice root and extracts thereof as well as liquorice products [1,2].

Since there is no common and validated analysis method for determining OTA in liquorice root extracts and liquorice products, on behalf of CAOBISCO (Association of the Chocolate, Biscuit and Confectionery Industries of the EU, European Cocoa Association, Brussels) a European Proficiency Test was organised, coordinated and evaluated by the LCI. The aim of the proficiency test was, firstly, to review the performance of participating laboratories in OTA analysis conducted on liquorice products at European level and, secondly, to research the influence of the extraction solvent on the quality of the results.

A total of 15 laboratories experienced in mycotoxin analysis and located in eight European countries took part. In each case two liquorice powders and pastes naturally contaminated with OTA and pretested for sufficient homogeneity were examined. Analysis of the data was conducted in compliance with commonly used international statistical procedures [3].

The findings are presented.

References:

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The application of PCR for identification of *Fusarium culmorum* strains producing large and small amounts of deoxynivalenol

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Many of the *Fusarium* species, including *F. culmorum* produce phytotoxic metabolites – mycotoxins, which are also harmful for human and animals. The most common contaminant of cereals in Europe is belong to trichothecene B, deoxynivalenol (DON). It has been established that strains of *F. culmorum* are able to produce large or small amounts of DON. Identification of the strains is possible by measuring of DON content or by the application of the specific PCR. The aim of the study was to establish the type of the 51 polish isolates of *F. culmorum* isolated from cereals and amount of DON produced by them. The most of the tested isolates were classified by PCR as high DON producing strains (44) and only 7 isolates as low DON producers. The isolates were also separated into two groups by ELISA test as high and low DON producers. The comparison analysis performed with data provided by both PCR and ELISA resulted in a reproducible outcome.

An accelerated method for determining sphingoid bases in serum of pigs

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Introduction: The ratio of the sphingoid bases sphinganine (Sa) to sphingosine (So) in plasma or serum is a biomarker for the consumption of the mycotoxin fumonisin B1. The commonly used strategy to determine the Sa to So ratio is to extract sphingoid bases, to subsequently cleave phosphorylated Sa and So by base hydrolysis and to quantify both originally free and originally phosphorylated sphingoid bases [1,2]. However, this approach requires tedious washing and two evaporation steps. The aim of the present work was to speed up the sample preparation procedure and to develop a rapid HPLC-FLD method for subsequent analysis of Sa and So.

Method Description: 200 µl of serum sample are incubated with 1.3 ml of methanolic KOH (0.154 M) and 0.4 ml of chloroform in the presence of C17-sphingosine as internal standard under shaking for 1 h at 37°C. Subsequently, 1.2 ml of chloroform are added and the solution is washed 3 times with 2 ml of water containing 0.8 mM ammonium hydroxide (pH 8-10). After that, the chloroform phase is evaporated to dryness, the residue is derivatized with OPA-reagent according to [1], the solution is centrifuged and the supernatant is used for HPLC-FLD analysis (excitation at 335 nm, emission at 440 nm) under the following conditions: 25 µl of derivatized standards or sample extracts are injected and separated on a C6 phenyl column (50 x 2 mm, 5 µm, Phenomenex) at 0.5 ml /min using gradient elution. Mobile phase A is 0.1% glacial acetic acid in deionized water, mobile phase B 0.1% glacial acetic acid in methanol. The gradient is the following: 0 min: 75% B, 4.5 min: 85% B, 6.8 min: 85% B, 7 min: 75% B, re-equilibration until 10 min.

Results: The limit of detection (S/N = 3) of Sa and So in standard solutions was 0.2 ng/ml, the limit of quantitation in serum of pigs was 1.6 ng/ml. These values are below the average level of Sa (17 ± 15 ng/ml) and So (105 ± 86 ng/ml) in serum of 29 untreated pigs as reported by [1]. The recovery was 75% for So, 91% for Sa and 86% for the internal standard C17-So. Calibration functions were linear up to at least 5000 ng/ml. The relative standard deviation (N = 5, one day) of analyte peak areas in a standard solution containing 0.1 mg/l of each compound was ≤ 4.2%. With the newly developed method it is easily possible to work-up and analyse 20 samples per day.

References:

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Ochratoxin A metabolites in human serum and urine: Implications for biomonitoring of ochratoxin A

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Ochratoxin A (OTA), mainly produced by *Aspergillus* and *Penicillium* species, is a frequent contaminant in foods and feedstuffs. Biomonitoring methods have been used to determine internal exposure resulting from dietary mycotoxin intake and inhalation of OTA-containing dusts in humans [1,2]. Levels of OTA in blood and/or urine samples provide good estimates of past and recent exposure since OTA binds to serum proteins and is also partly excreted via the kidney. But, measuring OTA (parent compound) alone does not reflect its biotransformation in the organism and at present information on OTA metabolites in human samples is scarce.

The aim of this study was to develop a method that allows OTA-analyzing as well as its major metabolite OTα in urine and in plasma samples. The method involves liquid-liquid extraction with chloroform and subsequent analysis by HPLC with fluorescence detection. The method has been validated for both matrices: The limit of detection and quantification for OTA and OTα are 0.08 and 0.1 ng/ml in plasma, and 0.02 and 0.05 ng/ml in urine, respectively. The RSD within-day and between-days shows values below 10% at different spike levels. The mean recoveries were 61% and 94% for OTα and OTA in urine, and 106% and 101% in plasma samples. The method was applied in a pilot study with volunteers (7 males, 7 females) who provided blood and urine samples on the same day. OTA and OTα were detected in all samples; the average OTA concentration in males (0.26 ng/ml plasma) was slightly higher than in females (0.23 ng/ml plasma), and average OTα levels in plasma were 0.1 and 0.08 ng/ml in males and females. Thus, OTA is the predominant form in blood, with OTα as main metabolite. Conversely, OTA concentrations in urine samples (average 0.07 ng/ml) are much lower than in blood, whilst the metabolite OTα is the predominant form in urine (average 1.24 ng/ml). This study implies that measuring OTA alone may underestimate mycotoxin exposure when metabolites are not analyzed in parallel, and that biomonitoring, especially in urine samples, is improved by including OTα.

References:

[1] Scott PM (2005) Biomarkers of human exposure to ochratoxin A. Food Additives and Contaminants 1: 99-107
[2] Degen GH, Mayer S, Blaszkewicz M (2007) Biomonitoring of ochratoxin A in grain workers. *Mycotoxin Research* 23: 88-93

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Development of cost effective analytical method for the determination of biomarkers (aflatoxin M_1, ochratoxin A) in human urine

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Aflatoxins (AFs) and ochratoxin A (OTA) are important and frequently occurring mycotoxins. Their acute and chronic toxic effects are well known [1-2]. The evaluation of the possible risk of aflatoxin B_1 (AFB_1) and OTA to human health requires to estimate their daily intake by the population or to determine their biomarkers in biological materials, e.g. blood serum, urine [3]. The presence AFB_1 and OTA biomarkers has been systematically monitored in biological materials (e.g. human blood serum, human urine) in the Czech Republic [4].

The aim of this study was a development of cost effective analytical method (cost x benefit) for the determination of biomarkers (AFM_1, OTA) in human urine (24-hour collection).

The cleaning of AFM_1 and OTA in urine (twenty ml) was carried out using immunoaffinity columns AFLAPREP® M and OCHRAPREP® (r-Biopharm Rhone).

The modified EIA methods (RIDASCREEN® Aflatoxin M_1, RIDASCREEN® Ochratoxin A) and the chromatographic methods (HPTLC, HPLC-FD, LC-MS/MS) were employed for purposes of the AFM_1 and OTA detection and quantification. The limits of quantification (LOQ) of the analytical methods are presented in the table.

Table: The limit of quantification (LOQ) of the methods

Methods	Biomarkers	
	AFM_1 LOQ (*ng/L*)	OTA LOQ (*ng/L*)
EIA	0.125	0.625
HPTLC	10	10
HPLC-FD	0.420	2
LC-MS/MS	0.6	0.7

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A rapid strip test for the quantitative determination of total FB fumonisins in maize

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Fumonisins are mycotoxins produced by several *Fusarium* spp. Of the group B fumonisins, FB1, FB2, and FB3 are the most relevant found in naturally contaminated food and feed such as maize. These secondary metabolites are known to be the causative agents of equine leucoencephalomalacia and porcine pulmonary edema. Furthermore, FB1 is a group 2B carcinogen.
A one-step lateral flow test was developed for the determination of total FB fumonisins in the range of 500-5000 µg/kg in maize, covered through performing multiple dilution steps of the sample extract. The presented quantitative test gives a result within 5 min including 3 min test and a 1 min strip drying and does not require any sample clean-up steps after a 3 min extraction. The extraction can be performed with water, methanol:water (70:30, v/v), or ethanol:water (70:30, v/v), respectively. A quantitative measurement is performed with a compact photometric strip reader (XReader, Romer Labs) which allows measuring up to four strips simultaneously. The test is based on a competitive immunoassay format where an antibody-colloidal gold particle complex is mixed with 50 µL sample extract in a microwell and used as signal reagent. The strip test is inserted into the well and the mixed content migrates onto the strip, which contains a test zone and a control zone. Mycotoxin-protein conjugate coated on the test zone captures free signal reagent, and colour particles concentrate forming a visible line. The intensity of the test line is dependent on the total FB fumonisin concentration in the sample. Naturally contaminated quality control maize material was used for matrix-matched calibration. The presented test is both quantitative and rapid with no cross-reactivity to other mycotoxins.

Aflatoxins in palm kernel cake from Indonesia – Applicability of ELISA in contrast to HPLC

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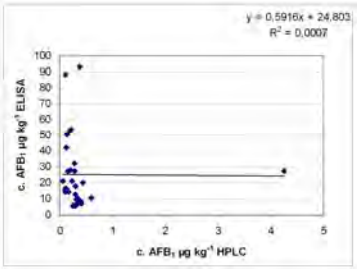
Introduction: Aflatoxin contamination has been well known as a world-wide health-threatening problem in tropical countries including Indonesia. Within a 6th frame work project of EU (Biotracer) samples were analysed for aflatoxins (AFs) indicating high contamination levels in palm kernel cake (PKC) from Indonesia. PKC samples were screened using an ELISA test kit and verified by HPLC-FLD analysis after immunoaffinity columns (IAC) clean-up.

Materials and Methods: For ELISA sample preparation and detection were carried out using procedure mentioned in the manual of ELISA kit from r-biopharm (Ridascreen). The sample clean-up using immunoaffinity columns IAC) has been performed after some modifications of washing steps to eliminate interfering matrix peaks in chromatograms. AflaCLEAN™ IACs were purchased from LC-Tech (Dorfen, Germany). The HPLC analysis of the AFs was carried out using an isocratic system. The signal enhancement was performed by Kobracell.

Results: It was necessary to optimize the IAC procedure for this special matrix. The whole analysis method was validated then for PKC prior to analyse samples. PKC extracts were spiked with a defined amount of AF and applied on the IAC. By applying the proposed procedure interfering matrix peaks appears in chromatograms. Therefore, washing step in clean-up had to be optimized. Spiking extracts of PKC samples with AFs showed recoveries ranging from 98-107% for the aflatoxins G_2, G_1, B_2 and B_1 respectively. 32 PKC samples from different batches were analysed for AFs using ELISA method. The results showed levels between 5.7 and 93.1 AFB_1 µg/kg. In contrast HPLC analysis delivered results between 0 and 4.3 µg/kg AFB_1. Additionally no correlation between the results obtained by ELISA and HPLC was found. In the following table mean value, median, maximum and minimum values are presented.



	$X_M AFB_1$ (µg/kg)	median AFB_1 (µg/kg)	max AFB_1 (µg/kg)	min AFB_1 (µg/kg)
ELISA	25,1	16,4	93,1	5,8
HPLC	0,4	0,2	4,3	0,0

It has been shown, that ELISA is not an appropriate method for the determination of high coloured sample extracts such as PKC extracts.

Cross reactivity in some commercial deoxynivalenol kits against some structurally related compounds

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Introduction: Co-occurrence of deoxynivalenol (DON) with other DON derivatives (3- and 15-acetyl DON) or other *Fusarium* toxins, including T2-toxin, HT2-toxin, fusarenon X (Fus X), diacetoxyscirpenol (DAS), nivalenol (NIV) is a potential mycotoxin concern for European cereals and cereal products. Plants infected by toxigenic fungi, can partly convert some mycotoxins. It was described that DON can be converted into DON-3β-glucopyranoside (D3G) in wheat as detoxification product. Antibody-based techniques provide a simple and economical alternative to instrumental methods for mycotoxin analysis. It should be mentioned that knowledge of assay specificity (cross reactivity) is critical for correct data interpretation since most immunoassay for trichothecenes have moderate or even strong cross-reactivity with closely related analogues. As National Reference Laboratory (NRL) for mycotoxins in Belgium, CODA-CERVA, has been appointed to perform an evaluation work on the technical quality (including cross reactivity) of kits commercially available in Belgium for determination of mycotoxins in food and feed.

Materials and Methods: Three commercial ELISA (i) AGRAQUANT® (Romerlabs), (ii) DON EIA (Eurodiagnostica) and (iii) Veratox® for DON 5/5 (Neogen Corporation) were tested according to the assay procedures recommended by kit providers. External standard of DON (not provided by the manufacturer) and the following mycotoxins (nivalenol (NIV), fusarenon X (FUS X), verrucarol (VCL), zearalenone (ZEA), T2 toxin, HT2-toxin purchased from Sigma Chemical Co., de-epoxyDON (DOM-1), D3G, 3AcDON, 15AcDON, and Diacetoxyscirpenol (DAS) obtained from Biopure) were used. Measurements of cross reactivity were carried out in 8 concentrations (2 replications) in aqueous solution (range: 0.0 – 100 ng/ml) at concentrations corresponding to approximately 50%, B/Bo (relative cross-reactivity, CR %).

Results: The results obtained on 11 compounds indicated that antibodies used in Agraquant, DON EIA and Veratox kits have high relative cross reactivity against 3ACDON, DOM-1 and D3G in various ranking orders. Moreover, DON EIA shows cross-reactivity against NIV and Fus X. Low or absence of cross reactivity were diversely observed for other mycotoxins such as 15-AcDON, DAS, T2-toxin, HT2-toxin, VCL and ZEA within the range of the tested concentrations. Some of these mycotoxins could coexist in cereal products and other food or feedstuffs. So, analytical results produced by these ELISA kits can be wrongly interpreted.

Discussion and Conclusion: High cross reactivity is considered as a drawback because it does not allow checking the compliance with the legal norms. Nevertheless, it may allow giving an idea about the whole amount of the target compound together with other detectable analogues. Data obtained in this work are somewhat different from the declared values by the manufacturers. Hence, there is a need to update regularly this information. For the validation of rapid tests, we suggest to standardize the choice of mycotoxins and metabolites to be tested in cross reactivity experiments according to the nature of the target analyte. As for DON kits, 3ACDON, NIV, D3G, DOM-1 and FUS X should be included. This may help in improving the quality of data obtained with such kits.

Development of a semi-quantitative rapid strip test for T-2 toxin and HT-2 toxin in wheat and oats

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Mycotoxins are toxic natural secondary metabolites produced by several species of fungi on agricultural commodities during storage or in the field.
The T-2 and HT-2 toxins, which are toxic for humans and animals, are type A trichothecenes produced by *Fusarium* species. Rapid immunoassay-based tests such as lateral flow devices (LFDs) for mycotoxins are increasingly being used. LFDs allow qualitative or semi-quantitative determination of mycotoxins within a few minutes and will have a major part in the feed and food sector besides standard analytical methods such as LC-MS.

The test strip is based on a competitive immunoassay format in which a gold labelled antibody is used as signal reagent. The colloidal gold particles with a diameter of approximately 40nm are prepared with controlled reduction of gold chloride. A mix which includes the gold labelled antibody is diluted in a microtiter plate with a wheat or oat extract. These extracts are obtained by shaking at a ratio of 1:5 for three minutes on an overhead shaker followed by filtration. After insertion of a strip into the well, the mix flows along the membrane of the strip, which contains a test line, where a mycotoxin-protein conjugate has been coated and a control line, which confirms correct test performance. The mycotoxin-protein conjugate captures the free antibody-colloidal gold particle complex, allowing colour particles to concentrate and form a visible line. The intensity of the line, which depends on the analyte concentration, can be measured with a photometric reflectance strip reader. Results are obtained within five minutes.

A major part of the presented work was the improvement of strip test materials and reagents such as membrane type and treatment and T-2/HT-2-protein conjugates. Also, different extraction solvents and extraction ratios were tested. The strip test was calibrated with spiked wheat and oat blanks for a semi-quantitative screening of total T-2 and HT-2 toxin.

The developed lateral flow device showed a linear working range of 0-1000 µg/kg in oats and wheat which was used for the semi-quantitative determination of total T-2 and HT-2 toxin.

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Immunochemical assessment of alternariol in food

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Introduction: Alternariol (AOH) is a mycotoxin produced by different species within the genus *Alternaria*. AOH was found to be a natural contaminant in cereals, fruits, vegetables, and products thereof (1). However, data on AOH occurrence in food and feed are scarce. Chromatographical methods used for determination of AOH are time-consuming, expensive and generally have a relatively low sample throughput. The objective of the present study was to determine the presence and concentration of AOH in food using a competitive indirect enzyme immunoassay (EIA) with polyclonal antibodies. This test was applied for the analysis of AOH in fruit juice. For analysis, samples were diluted with buffer solution, without any cleanup steps necessary. The method has a reasonable limit of detection, for instance 1 ng/ml for fruit juice. The EIA is very specific for AOH. The cross-reactivity with altenuene is 0.5 %, with alternariol monomethylether 0.2 %, and with tenuazonic acid less than 0.1 %. Further work will focus on the analysis of more sample matrices and on the optimization of the sample preparation.

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A new multiplex immunoassay format for mycotoxin analysis based on Luminex xMAP technology

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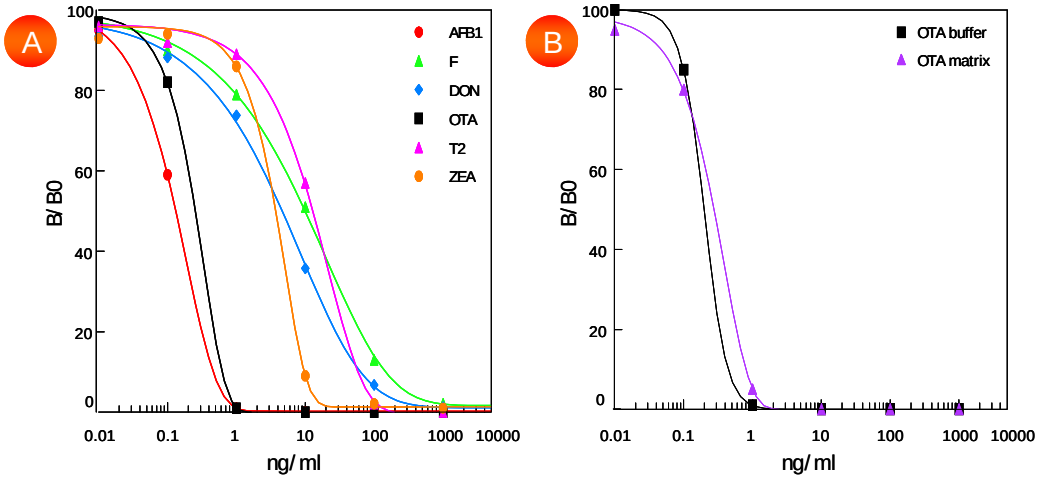
Introduction: Mycotoxin detection in food and feed is one of the tasks of RIKILT and, so far, a method based on LC-MS/MS is used. Our research is focused on the development of a faster and cheaper alternative method using the Luminex xMAP multiplex technology and aiming for a combined competitive inhibition immunoassay for the detection of Aflatoxin B1 (AFB1), Deoxynivalenol (DON), Zearalenone (ZEA), T2-toxin (T2), Fumonisin (F) and Ochratoxin A (OTA) in feed extract.

Materials and Methods: The Luminex system is a small microsphere dedicated flow cytometer. The microspheres (5.6 µm) are internally dyed with precise ratios of two fluorophores to create 100 unique microspheres. Each microsphere can be distinguished based on its internal dye ratio using a red laser for excitation. Antigens are coupled to the carboxylated microsphere surfaces which react with the monoclonal antibodies in solution. After the microspheres are classified, the reporter signal is measured. Reporter antibodies (anti-mouse) are labelled with a fluorochrome (e.g. R-Phycoerythrin (PE)) and are excited with a green laser. In the present research, six different immunoassays were combined.



Results: After extensive testing, six antibodies were selected to set up a mycotoxin multiplex assay. The combination of these antibodies showed good calibration curves (figure A) when measuring all six mycotoxins multiplexed in buffer. In matrix extracts spiked with OTA the calibration curves were almost identical to the one in buffer (figure B).

Conclusion / Discussion: The developed assay allows multiplexed detection with high sensitivities ranging from 0.05 to 10 ng/ml in buffer. The assay can be performed within 2 hours. The assay has to be tested for measuring naturally contaminated samples. Success will be largely depending on the presence or absence of matrix effects in combination with the applied extraction method. For this purpose we will compare pre and post spiked extracts as well as naturally contaminated mycotoxin samples that are already analysed with the LC-MS/MS method.

Production and characterization of antibodies against fumigaclavine A

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Aspergillus fumigatus produces a number of secondary metabolites which could be addressed as mycotoxins because of their toxicity to humans and animals (1). Among others, *A. fumigatus* produces several clavine alkaloids (containing the ergoline ring system, the basic structure of ergot alkaloids), including fumigaclavines A, B, and C (2,3). Fumigaclavine A indirectly affects the peripheral and central nervous system (4). The aim of this study was to develop high-affinity antibodies against fumigaclavine A, to establish an enzyme immunoassay (EIA) for trace level detection of fumigaclavine A. A Mannich condensation reaction was used to conjugate fumigaclavine A to keyhole limpet hemocyanine (KLH) for immunization, and to bovine serum albumin (BSA) for use as the solid phase antigen. Three rabbits were immunized by using multisite intradermal injections. Booster injections, using the same composition and amount of immunogen, were given subcutaneusly or intramuscularly. To characterize selected antibodies for fumigaclavine A, a competitive indirect EIA was established.Results indicate that the antiserum obtained from one rabbit has a high affinity to fumigaclavine A, with 50% inhibitory concentration (IC_{50}) values ranging from 2 to 3 ng/ml. The tests performed so far indicated that the rabbit antibodies were specific for fumigaclavine A, and did not cross-react with several other ergot alkaloids, including ergonovine, ergotamine and beta-ergocryptine.

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Production and characterization of antibodies against tenuazonic acid

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Tenuazonic acid (TEA) is a mycotoxin produced by different species within the genus *Alternaria*. TEA was found to be a natural contaminant in cereals, fruits, vegetables, and indoor environment. However, only few data exist on the occurrence of TEA in food and indoor environment. Most of the methods used for TEA determination are chromatographic methods that are quite time-consuming and expensive. The objective of the present study was the production and characterization of polyclonal antibodies against TEA and the development of a sensitive enzyme immunoassay (EIA) for the determination of this toxin in different commodities.

TEA was derivatized with succinic anhydride. The TEA-hemisuccinate was then conjugated to keyhole limpet hemocyanin (KLH) as the immunogen, and to horseradish peroxidase (HRP) as the labelled antigen. Three rabbits were immunised with TEA-KLH conjugate, each of them was injected intradermally with 2 mL immunogen. After the basic immunisation, two restimulations followed at a time interval depending on the individual development of the specific antibody titer. The immune response was screened in a competitive EIA using anti-rabbit IgG in a double antibody solid phase system. The antibody specificity was determined by competitive binding inhibition of several other compounds compared with TEA standard solution.

The TEA-KLH conjugate demonstrated an immunogenic activity. Four weeks after the first immunisation, antibody titers could be detected in all three rabbits but only those of one rabbit were sufficiently specific after the first restimulation. The direct EIA systems established so far had detection limits of TEA standard solutions at levels of 50-200 ng/mL, and 50% inhibition values of 200-400 ng/mL. The EIA was highly specific for TEA and TEA-hemisuccinate and had negligible cross-reactivities with other *Alternaria* toxins.

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Evaluation of NeoColumn™ T2/HT2

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Introduction: NeoColumn™ T2/HT2, a new immunoaffinity column (IAC) for the detection of *Fusarium* spp. toxins T2 and HT2, was developed and evaluated at Neogen Europe Ltd. Alongside the IAC work, a simple isocratic HPLC method was developed for the detection of T2/HT2. An extensive evaluation programme has demonstrated that NeoColumn T2/HT2 is a highly effective IAC, which can recover T2 and HT2 from a broad range of matrices including horse feed, wheat and oats. Data show excellent levels of recovery from samples and that the column is a useful analytical tool for the food industry. In view of imminent EU legislation, NeoColumn T2/HT2 is a timely addition to the current range of NeoColumns.

Materials and Methods: Two unique monoclonal antibodies exhibiting 100% cross reactivity for T2 and HT2 toxins, were immobilised to a custom-made resin. Using a process of spiked and naturally contaminated sample evaluation, the IAC was optimised for T2 and HT2 toxin recovery. The isocratic HPLC method was developed using a 5µm, 150 x 4.6mm HPLC column and adapted from the method proposed by Visconti *et al*. Samples were subjected to pre-column derivatisation prior to assay.

Results: The data show that T2 and HT2 can be recovered from a variety of matrices, whether spiked or naturally contaminated. The isocratic HPLC method of analysis was shown to be robust and simplifies the procedure for the detection of T2/HT2.

Discussion and Conclusion: NeoColumn T2/HT2 is a robust IAC that is highly effective for use in the detection of T2 and HT2 in a variety of matrices.

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Visconti, A.; Lattanzio, V.M.T.; Pascale, M.; Haidukowski, M., 2005. Analysis of T-2 and HT-2 toxins in cereal grains by immunoaffinity clean-up and liquid chromatography with fluorescence detection. J. Chrom. A, 1075, 151-158.

Occurrence of deoxynivalenol in wastewater

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For several years, our research group has focussed on the occurrence of mycotoxins in the aquatic environment. We were able to describe the route of zearalenone (ZON) from *Fusarium* infected plants (wheat, maize) to the soil, and into drainage water [1]. Since deoxynivalenol (DON) usually exceeds ZON concentration in wheat and maize by least ten times, and because it is much more water soluble, we started the quest for DON in water samples. Indeed, DON emissions by drainage water surpassed those of ZON by a factor of about 200. DON was also present in a majority of water samples from the river Glatt and Töss in July and August 2007 [2]. However, the question remained whether *Fusarium* spp. infected crop fields were the only source of DON. We therefore decided to open up our perspective and adapted our analytical LC-MS/MS method to samples originating from wastewater treatment plants (WWTP). While testing the new method it became apparent that the use of isotope-labelled compounds as internal standards is mandatory. Not only because effluent samples of the primary and the secondary clarifier showed different matrices, but also because the origin of sewage water for each WWTP is not comparable. We will present first results on the occurrence of DON in flow proportional composite effluent samples of the primary and secondary clarifiers of three different WWTP in Switzerland. Our data matches very well with predictions based on urinary excretion of DON [3,4].

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Toxigenic fungi and mycotoxins in house dust

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Spores and hyphae-fragments of filamentous fungi are a natural component in house dust and derive from indoor and outdoor sources. Among them are potentially toxigenic fungi. The aim of our study is to investigate whether an indoor mould infestation leads to a higher content of spores and/or mycotoxins in house dust.

For this purpose we collected 150 dust samples from households with known mould infestation and another 150 from control households without mould. To determine the concentration and species of viable spores dilution series with sieved dust (≤ 63 µm) were prepared and applied on maltextract- and DG18-agarplates (Toepfer and Butte 2009). After incubation the species were determined morphologically. The following 8 mycotoxins were quantified by tandem mass spectrometer (LC-MS/MS) in the multiple-reaction-mode: aflatoxin B1, deoxynivalenol, diacetoxyscirpenol, citrinin, gliotoxin, ochratoxin A, roridin A and sterigmatocystin.

The concentration of viable spores reached from 9,000 up to 164,000,000 cfu (colony forming units) per g house dust. There existed no association between the spore concentration and the occurrence of a mould infestation in the house.
Aspergillus versicolor is a primary colonizer on damp building materials and is able to produce a high yield of sterigmatocystin at a water activity close to 1 (Nielsen 2003). *A. versicolor* could be detected in 68 % of the dust samples ranging from 1,000 to 26,000,000 cfu/g with a median of 9,000 cfu/g (on DG18-agar). Sterigmatocystin was found in only a few samples from either type of household. The concentrations were in the lower µg/kg range. Not all samples containing sterigmatocystin showed high concentrations of viable spores of potential producers like *A. versicolor*. A similar observation was made with other detected mycotoxins.
The lacking association between a mycotoxin concentration and the detectable content of the corresponding producers can be explained by the fact, that mycotoxins are not only released from viable spores but from non viable colony fragments as well (Nielsen 2003). But these particles are not detected by plate count. With other methods e. g. microscopy or analysis of chemical indicators, which detect viable and non viable parts of fungi, one cannot determine the species and their capability of mycotoxin production.

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Assessment of a multi-mycotoxin contamination of maize and maize products

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This project focused on the assessment of a multiple mycotoxin contamination of maize and maize products. By means of a newly developed LC-MS/MS multi-method, 155 representatively collected samples (maize flour and semolina, maize pasta, breakfast cereals, and others) were analysed for the occurrence of trichothecenes, fumonisins (FUM), zearalenone (ZEA), aflatoxins, ochratoxin A (OTA), and mycophenolic acid. Furthermore, DON-3-glucoside and the hydrolysis products of fumonisin B1 were included into the study.

The number of simultaneously detected mycotoxins ranged between 2 and 20 toxins per sample. The results were evaluated in detail with respect to the six most important analytes/groups of analytes (T-2/HT-2, DON, FUM, ZEA, aflatoxins, and OTA). Only six samples (3.9 % of all samples) contained one toxin of these groups. More often, four of these types of mycotoxins were detected simultaneously (in 40 % of all samples). A majority (84 %) of these combinations of analytes included T-2/HT-2, DON, FUM and ZEA. However, only two samples (1.3 %; maize pasta with Italian origin) were contaminated with all six types of toxins.
The concentrations of DON, ZEA, FUM ranged from not detectable to 1275, 190 and 1025 µg/kg, respectively. In some samples also aflatoxins were detected up to 2 µg/kg (sum of aflatoxins).

Finally, the estimation of mycotoxin intake resulted in a low risk to exceed the established TDI-values for all consumers; for people with a high maize consumption (e.g. patients suffering from celiac disease), however, the multiple contamination of maize products may potentially impose a risk, especially with regard to children.

Bran samples from the European market: Levels of Deoxynivalenol, Zearalenone, Ochratoxin A, T2- and HT2-Toxin and occurrence of microorganisms

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The outer layers are the part of the grain containing important health-improving and disease-preventing substances, for example fibres and phytochemicals such as lignans, vitamins and phenolic compounds. Therefore, whole grains and also brans are universally recommended as part of a healthful diet. The outer layers are also the part of the kernel contaminated the most with mycotoxins originating from field fungi. Focussed on healthy nutrition, we analysed 46 bran samples from the European market for their mycotoxin content and the occurrence of microorganisms.
The results showed that in 13 percent of the samples no mycotoxin could be detected, whereas more than a quarter of all bran samples exceeded the Deoxynivalenol maximum level of cereals for direct human consumption according to the European Union regulation 1881/2006/EC. Zearalenone was found in nearly 90 percent of all samples but none exceeded the maximum level given in 1881/2006/EC. In one bran sample HT2-Toxin was found. The mycotoxin Ochratoxin A, typically synthesised by fungi during storage of products, could not be detected in more than 70 percent of the samples. Total bacterial and fungal counts were generally well below guidance levels for cereal products.
Using bran, our results show that a high quality of food stuffs is not only determined by nutritionally favourable substances but also by absence or low levels of contaminants, in this case mycotoxins. Concerning consumer protection it is important to choose high quality raw materials to produce healthy and safe bran and bran products.

Characterization of *Alternaria alternata* strains isolated from Czech foodstuffs

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Microfungi of the genus *Alternaria* are ubiquitous pathogens and saprophytes. Many species of the genus *Alternaria* commonly cause spoilage of various food crops in the field or post-harvest decay. *Alternaria alternata* is a frequently occurring species and probably the most important mycotoxin-producing species that occurs on cereals, sunflower seeds, oilseed rape, tomato, carrot, lentils, olives, various fruits etc. *A. alternata* produces a number of mycotoxins, including alternariol, alternariol monomethyl ether, altenuene, altertoxins I, II, III, L-tenuazonic acid and other less-toxic metabolites [1].

The aim of this study was a mycological, molecular biological and toxicological characterization of *A. alternata* strains isolated from Czech winter wheat (34 strains), grapes (11 strains) and tomato (1 strain).

All the *A. alternata* isolates were studied in culture and identified according to Simmons [2]. Within the *A. alternata* strains are defined primarily upon conidium characteristics including size, septation, presence and/or size of a beak, and pattern of catenation.

All the *A. alternata* strains were tested by PCR method (target sequences – ITS region ribosomal DNA) [3].

All the *A. alternata* strains were cultured on rice to evaluate their toxigenic potential, because rice was described as suitable for *Alternaria* toxin production [4]. An HPTLC method for quantification of *Alternaria* toxins was used [5].

We discovered the differences between *in vitro* toxigenity potency of *A. alternata* strains isolated from Czech foodstuffs and the occurrence of *Alternaria* toxins in these foodstuffs.

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Hygienic quality of green forage and silage from maize contaminated with *Ustilago maydis*

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In the last years there has been an increase in the cases of maize contaminated with smut. The toxicity of the smut is not well known. Therefore, the aim of the study was mycological and mycotoxicological evaluation of the contamination of green forage and silage from maize contaminated with *Ustilago maydis.*

The samples of the contaminated maize (*Zea mays* L.) REDUTA (FAO 230) were taken from ZDD Swadzim (2008), the field of 0,15 ha, namely green forage from the whole plant at the wax phase of growth (September) and ears at full maturity (October). Maize that was not infected with the smut, harvested from the same field was used as the control samples. The silages were made in 4 dm^3 capacity microsilos. Hygienic evaluation and mycotoxins content were done for the raw material and silage. The general number of fungi and mycotoxins content were determined. Samples, initial suspention and ten-times dilutions used for microbiological analysis were carried out according to PN–EN ISO 6887–1, July 2000. The general number of moulds and yeast (cfu/g DM) was determined according to PN–ISO 7954, September 1999 (with own alternation). Cytotoxicity level was determined by the MTT test with swine kidney cells (SK), sensitive to a wide range of mycotoxins. The extraction of mycotoxins was done on the immunoaffinity columns: AflaTest and FumoniTest by Vicam for AF and FUM respectively, Ochraprep by r-Biopharm Rhône Ltd for OTA and Bond Elut® Mycotoxin (Varian) for trichothecens and zearalenon. Mycotoxins were evaluated with the HPLC method (Merck-Hitachi, Agilent) with fluorescent detection (OTA, AF) and with the HPLC-MS/MS method (3200 QTRAP, Applied Biosystem) for trichothecenes, ZEA and FUM. Cobra Cell was used for aflatoxins derivatization.

The number of lactic acid bacteria was determined according to the horizontal standard PN–ISO 15214, August 2002 (with own alterations). The number of mesophilic bacteria was determined according to the standard PN-R-64791, December 1994 (with own alterations).The content of the selected mycotoxins is presented in the table. Neither AF nor OTA was detected in the samples. However, it was found that during the fermentation process the level of DON and ZEA increased significantly with distinct reduction of the number of moulds. High level of FUM was detected in the smut infected maize as well as high level of moulds contamination $>10^8$cfu/g DM, and the dominating species was *Fusarium moniliforme*. Moreover, high levels of mesophilic and lactic acid bacteria were also detected in the samples examined. The results of the MTT test showed high cytotoxicity of the samples of smut infected maize. Such maize may pose a health hazard to animals.

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Presence of ochratoxin A in biological fluids from lactating mothers and correlation with levels in urine from their babies

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Ochratoxin A (OTA) is an important mycotoxin with well documented occurrence as contaminant in animal feeds and human food. Ingestion of OTA-contaminated food results in the presence of this mycotoxin in different biological fluids, i.e. urine, plasma, and human milk [1, 2]. OTA levels in these samples can vary considerably with exposure and for different geographical areas [3]. As infants may be more susceptible to adverse effects of toxicants than adults, with mother's milk being often the main food for neonates and babies, further studies are indicated to assess OTA exposure in this setting.

It is the aim of an ongoing study to assess OTA exposure of babies from mothers' milk and to characterize the lactational transfer of OTA in mothers (blood/milk ratio). So far, 9 mothers were asked to provide plasma, urine and milk samples and urine from their babies. The first samples were collected at the hospital until two days after delivery.

We use liquid-liquid extraction with chloroform for sample clean-up and HPLC with fluorescence detection for subsequent analysis of extracts. The methods for OTA analysis have been adequately validated for all three matrices and are suitable for sensitive biomonitoring. The limits of detection (LOD) for OTA are: 0.08 ng/ml in plasma and 0.02 ng/ml in urine and in milk samples. The results for OTA levels determined in mother's blood and plasma as well as first data on OTA in infant urine will be presented and discussed.

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Moulds and mycotoxins in commercial dry dog and cat food

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Introduction: The presence of cereals and grains in the formulation of pet foods suggests the need to control moulds and mycotoxins contamination in these foods. Mycotoxins may have chronic effects on the health of companion animals. The objective of this study was to investigate the occurrence in dry pet (cat and dog) food of moulds and mycotoxins: aflatoxins (AF), ochratoxin A (OTA), deoxynivalenol (DON), T-2 toxin, HT-2 toxin, zearalenone. Additionally, samples containing maize were tested for fumonisins (FUM).

Materials and Methods: 49 packets of dry food: 24 of cat food (including 9 for kittens and 15 for cats) as well as 25 of dog food (including 5 for puppies and 20 for dogs) were purchased. Out of these, 23 packets were produced in Poland, 23 in EU while 3 in other countries.
Quality and quantity identification of moulds and yeast was done in accordance with PN ISO 7954: 1999 – with own alternation (inoculation on YGC medium – yeast extract, glucose, 100 ppm chloramphenicol, incubation 5 – 7 days in 25 °C).
The extraction of mycotoxins was done on the immunoaffinity columns: AflaTest and FumoniTest by Vicam for AF and FUM, Ochraprep by r-Biopharm Rhône Ltd for OTA, for DON, T-2 toxin, HT-2 toxin and zearalenone Bond Elut® Mycotoxin columns were used. Mycotoxins were evaluated with the HPLC method (Merck-Hitachi, Agilent) with fluorescent and MS/MS (Applied Biosystems) detection. Cobra Cell was used for aflatoxins derivatization.

Results: In the examined samples high levels of neither moulds nor yeast were detected (maximum 250 cfu/g). The most commonly occuring fungi were *Aspergillus*, *Mucor* and *Penicillium* species. Characteristic is the fact that *Aspergillus fumigatus* dominated in cat food, while *A. nidulans, A.glaucus, A. flavus, A. niger* and *A. sydowii* were found in dog food.
Deoxynivalenol was detected in all the examined samples, at the average level of 97,3 ng/g and 114 ng/g (for cat and dog food, respectively); the highest detected concentration was 359 ng/g in cat and 436 ng/g in dog food. Zearalenon was detected in all the samples at the average level of 38,3 ng/g and 20,1 ng/g (in cat and dog food, respectively); while the highest detected level of zearalenon was 123 ng/g in cat and 75 ng/g dog food. T-2 toxin was present in 87,5% of cat and 88% of dog food samples, while HT-2 toxin was detected in 79% of cat and 88% of dog food samples. In cat food the highest detected concentrations of T-2 and HT-2 toxins were 11,5 and 12,0 ng/g, respectively; whereas in dog food 13,3 and 19,6 ng/g, respectively. Ochratoxin A was found in nearly half of the samples examined and its maximum concentration was 3,0 and 2,23 ng/g (in cat and dog food, respectively); the average value of all positive samples was 1,02 ng/g. Aflatoxins and fumonisins were present in single samples, where the highest concentrations of the sum of aflatoxins were 0,21 ng/g in dog food sample, and fumonisins 108 ng/g in cat food sample.

Multifactorial spatial analysis of mycotoxin contamination of winter wheat

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Toxin production by *Fusarium* species occurs mainly in the field. The production of DON and ZEA by *F. culmorum* and *F. graminearum* is greatly influenced by genetic, environmental and agricultural management factors. A variable factor for the fungi's development in cereals has been neglected: Soils and crops are not homogeneously distributed within a single field: These within-field variations can be observed in different yields, varied grain protein content, spatially variable ripening and increased fungal infection. Further, most agricultural landscapes in Germany show at least some relief. The resulting redistribution of water within landscape or single fields leads to spatially heterogeneous soil and air humidity. Because of higher fungi infection potential in more humid parts of a landscape or a field, these sites might show higher mycotoxin accumulation.

We studied the mycotoxin accumulation in winter wheat growing after maize or oilseed rape at different topographic positions within fields consisting of alternating hilltop and depression positions across a precipitation transect in the Uckermark region in the Northeast of German Lowlands in 2006 and 2007.

Environmental factors (actual rainfall during anthesis, long-term mean annual precipitation), topographic factors (relief position, topographic wetness index TWI) and management factors (tillage, preceding crop, wheat cultivars) as well as the year of investigation were included as explanatory factors in this calculation.

For data analysis we used a spatial linear mixed-effects model and censored regression techniques to account for spatial autocorrelation and data below the detection limit, respectively. We use both approaches and compare the results because a combined technique is currently not available.

The results indicate that DON levels on wheat fields with maize as preceding crop are almost one order of magnitude higher than in wheat grown after oilseed rape (significant at the 5% level). Tillage has a significant effect of a very similar magnitude. Areas classified as humid (mean annual precipitation > 550 mm) show a significantly higher DON contamination (3.7-fold increase) than the drier areas. Precipitation during anthesis and the susceptibility of wheat cultivar to *Fusarium* infection have no significant influence on DON in the spatial linear mixed-effects model. The censored regression detects a small, but significant influence of precipitation during anthesis on DON content (approximately twofold increase). TWI and topographic position (hilltop versus depression) show significant positive influences on DON: DON levels in a topographically lower position (depression sites with high TWI) are on average 86% higher than at topographically elevated sites (hilltop sites with low TWI).

Mycotoxin contamination of different cultivars of spelt (*Triticum spelta*)

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Spelt was grown in ancient times and now it is renewed in organic farming (Chrenkova et al. 2000). Quality estimation of spelt yield in aspect of mycotoxins content is very important regarding consumer health. Mycotoxins produced by fungi are the one of the most dangerous compounds which contaminates cereal products. The aim of this study was to determine the presence of mycotoxins in different cultivars of *Triticum spelta.* Wholemeal flour was analysed on the content of four mycotoxins: aflatoxin, ochratoxin A, T-2 toxin and deoxynivalenol. The presence of these mycotoxins was determined by use of the Enzyme-Linked ImmunoSorbent Assay (ELISA) method.
Among 20 of the examined cultivars of winter spelt, deoxynivalenol was identified in 6 samples (up to 0.3 mg/kg), T-2 toxin was identified in one sample in very low amount (below 75 µg/kg) while aflatoxin and ochratoxin A were not found. Among 3 of the examined cultivars of spring spelt deoxynivalenol, T-2 toxin, aflatoxin and ochratoxin A were not found. Deoxynivalenol was found in following winter spelt cultivars: *T. spelta* L. album, *T. spelta* BG, *T. spelta* BG 1166, *T. spelta*, Schwabenspelz and Francencorn. T-2 toxin was identified in *T. spelta* L. album BG 31 cultivar.

Reference:

Chrenkova M., Ceresnakova Z., Sommer A., Galova Z., Kralova V., 2000. Assessment of nutritional value in spelt (*Triticum spelta* L.) and winter wheat (*Triticum aestivum* L.) by chemical and biological methods. Czech Journal of Animal Science, 45, 133-137.

Mycotoxins and fungi contamination of feeds and their components in the years 2006-2008 in Poland

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The fungi and mycotoxin contamination of products intended for animal feeding constitutes an agro-economical and health problem. There is a strong need to tighten the worldwide and EU legislation, which requires more occurrence information from different regions.
The aim of the study was to collect data on fungi and mycotoxin presence in feedingstuffs and their components – available on Polish market. The research was carried out from 2006 to 2008. The material was divided into four groups: cereal grains (wheat, rye, barley, oats), corn grains, mixed feeds and silages. The following numbers of mycotoxins analyses were performed: aflatoxins – 457, ochratoxin A – 523, zearalenone – 637, deoxynivalenol – 757, fumonisins – 106, T-2 toxin – 237, HT-2 toxin – 192, and nivalenol – 58. The general number of fungi was determined in 528 samples.
The extraction of mycotoxins was done on the immunoaffinity columns: AflaTest®, FumoniTest™ and ZearalaTest® (Vicam) for aflatoxins (AF), fumonisins (FUM) and zearalenone (ZEA); while Ochraprep® and Donprep® (r-Biopharm Rhône Ltd) for ochratoxin A (OTA) and deoxynivalenol (DON). Yet, for DON, ZEA, T-2 toxin, HT-2 toxin and nivalenol (NIV) Bond Elut® Mycotoxin columns were also used. Mycotoxins were determined with the HPLC methods (Merck-Hitachi, Agilent) with fluorescent, UV and MS/MS (Applied Biosystems) detection. Cobra Cell was used for aflatoxins derivatization. Quality and quantity identification of moulds and yeast was done in accordance with PN ISO 7954: 1999 (inoculation on YGC medium – yeast extract, glucose, 100 ppm chloramphenicol, incubation 5-7 days in 25°C), with own alteration.
It was found that less than 10% of samples were contaminated with aflatoxins at low level (maximum 0,31 ppb). The percentage of OTA-positive samples decreased during those three years (2006 – 87%, 2007- 48%, 2008 – 36%), with the highest average$_{positive}$ value of 22,7 ppb (cereal grains 2007). The share of samples, in which ZEA was present was constant throughout the period (~70%) and its maximum average$_{positive}$ concentration came to 122 ppb (corn grains 2006). Similar situation took place in case of DON (~90% of positive samples), where 1130 ppb was the highest average$_{positive}$ value (corn grains 2006). Fumonisins were present in 91% of the analysed samples (in 2006 and 2007) and in 68% (in 2008), with the maximum average$_{positive}$ concentration of 2260 ppb (silages 2007). Among other trichothecenes (analysed since 2007) nivalenol was detected in ~90% of samples and its highest average$_{positive}$ value was 139 ppb (silages 2008). The average T-2 and HT-2 toxins contamination level of the material was 28 and 58%, respectively and the maximum average$_{positive}$ concentrations were: 45,7 ppb (T-2, corn grains 2007) and 45,6 ppb (HT-2, silages 2008). In seven samples the concentration of particular mycotoxins exceeded the guidance values relating to feedingstuffs [1]. In mycological study the analysed material was divided into 2 groups – with reference to proposed contamination limit of 10^5 cfu/g (the general number of fungi) [2]. In samples, which general number of fungi did not exceed the value of 10^5 cfu/g, the highest average$_{positive}$ number of fungi came to 0,29 x 10^5 cfu/g (corn grains 2007), whereas in the second group (>10^5 cfu/g) the value was 474 x 10^5 cfu/g (silages 2008). The fungi and mycotoxin contamination level of the analysed samples confirms the need of further monitoring of feeds and their components.

References:

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Mycotoxins in horse feed

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Reports concerning mycotoxins in horse feed are very rare and typically are restricted to fumonisins. To our knowledge, a broader and systematic survey on mycotoxins in commercial horse feed has never been published. This is somehow surprising, since fumonisins and other mycotoxins may have various adverse effects in equines. Furthermore, mycotoxin residues may also present a problem if the horse is considered as food-producing species. Several companies offer ready-to-feed premixes of cereals and various other ingredients, typically called “muesli”. Other products are sold as pelleted mixtures. Oats, maize, and barley are also sold as plain cereals (e.g. puffed or flaked). The aim of this study was to perform a representative mycotoxin survey in commercial horse feed as available on the German market.

A total of 62 samples of commercial horse feed preparations (“muesli” (n=40), pelleted feedingstuff (12), oats (n=4), maize (n=4), and barley (n=2)) were purchased from 21 different producers/distributors, typically in bags containing 20-25 kg each. Several kg from each bag were thoroughly mixed in a bucket, and about 1 kg was ground to a particle size of <1 mm in a laboratory mill. All samples were analysed by enzyme immunoassays. Analytes (detection limit, mean recovery) were T-2 + HT-2 toxin (T-2/HT2, 0.2 µg/kg, 97%; T-2, 0.1 µg/kg, 71%), zearalenone (ZEA, 5 µg/kg, 93%), deoxynivalenol (DON, 10 µg/kg, 84%), ochratoxin A (OTA, 0.2 µg/kg, 67%), fumonisin B1 (FB1, 2 µg/kg, 113%) and ergot alkaloids (generic test for total alkaloids “GEA”, 30 µg/kg, 132%). All samples contained T-2, HT-2 and DON. T-2/HT-2 were detected in a concentration range of 0.8-228 µg/kg (median: 24 µg/kg), T-2 in the range of 0.3-91 µg/kg (median: 7 µg/kg). DON was found in a range of 16-4864 µg/kg (median: 219 µg/kg). ZEA was detected in 61 samples (98%) at concentrations of 7-312 µg/kg (median: 61 µg/kg). Most samples (n=58) were positive for fumonisins (range: 2-2188 µg/kg, median: 27 µg/kg). Ergot alkaloids were detected in 38 samples (61%) at concentrations of 28-1195 µg/kg (median: 97 µg/kg). OTA was found in 26 (42%) samples, at concentrations of 0.2-4 µg/kg (median: 0.35 µg/kg).

The results demonstrate that mycotoxins are omnipresent in commercial horse feed, exclusively as a multitoxin mélange. However, the toxin concentrations were in most cases relatively low, well below the levels which are usually considered as critical or even toxic. For example, all FB1 values were below the recommended maximum levels (5 mg/kg) in complementary and complete feedingstuffs for horses, and DON, ZEA, and OTA were mostly below the recommended levels in pig feed (European Commission Recommendation 2006/576/EC). In fact, nearly all of these materials would have been of “food quality”, since all seven toxins/toxin groups were present at levels well below the respective EU maximum levels for foodstuffs (DON, ZEA, FB1, OTA), or below the *de facto* accepted levels in foods (T-2, Ergot). However, at present very little is known concerning the chronic effects of these mycotoxins in horses, or concerning their carry over into edible tissues. Additionally, further studies concerning mycotoxin intake from non-commercial sources of horse feed seem to be advisable.

Mycotoxins in mare's milk

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Production and distribution of mare's milk is a small but growing market in Germany. The price of mare's milk is very high (>10 Euro per liter), and it is mainly sold via direct marketing channels as a kind of "therapeutic food product", using numerous health claims. In contrast to milk from other species, mare's milk typically is not heat treated, therefore specific regulations for certified raw milk (Vorzugsmilch) apply here. These include the necessity to analyse for relevant toxins and other contaminants. At present however, very little is known concerning the carry-over of mycotoxins from horse feed into mare's milk. Only one feeding study with aflatoxins has been performed so far (1) and indicated that the carry-over of aflatoxin B_1 as aflatoxin M_1 (AFM_1) in milk is about 0.05%, much less than in dairy cows. In the present study, we analysed 53 samples of mare's milk (tank milk) from 12 different producers in Germany by enzyme immunoassay after specifically designed sample preparation techniques. Analytical parameters (detection limit) included AFM_1 (5 ng/kg), ochratoxin A (OTA, 15 ng/kg), zearalenone (ZEA, 110 ng/kg), and T-2/HT-2 toxin (7 ng/kg). Additionally, deoxynivalenol-deepoxydeoxynivalenol (DON-DOM1) were analysed by HPLC-UV after glucuronidase treatment and immunoaffinity chromatographic sample cleanup (detection limit 1000 ng/kg each). AFM_1, OTA and ZEA were not detected. Traces of T-2/HT-2 (<50 ng/kg) were found in a few samples. Traces of DON (500-1000 ng/kg) were found in two samples, two other samples were positive for DON at a level of 2000-4000 ng/kg. Traces of DOM1 (1000 ng/kg) were found in one sample with the highest DON level. Since sample collection included the majority of mare's milk producers in Germany, these representative results indicate that the analysed mycotoxins are not a problem in mare's milk. The reason could be that either the carry-over of these toxins is low in horses, or that the mycotoxin levels in feed - and thus the toxin intake – were relatively low. The latter seems to be supported by another study (2) in which relatively low levels of these mycotoxins were found in commercial horse feeds from the German market. However, since the metabolism of horses strongly differs from that of ruminants, controlled feeding studies, and inclusion of other mycotoxins should be considered in further studies.

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Occurrence of aflatoxins in food in Lower Saxony in 2007 and 2008

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Introduction: Aflatoxins are mycotoxins produced by *Aspergillus flavus* and *A. parasiticus*. The Scientific Committee for Food expressed in its opinion of 23 September 1994 that aflatoxins are genotoxic carcinogens, aflatoxin B1 being by far the most toxic compound. The Commission of the European Communities set therefore maximum levels for the content of aflatoxin B1 alone and for the sum of aflatoxins B1, B2, G1 and G2 in groundnuts, nuts, dried fruits, cereals, maize, products thereof and spices. For Germany, maximum levels for aflatoxins in all other kind of foods are regulated in the so called Mykotoxin-Höchstmengenverordnung. In Lower Saxony, the Lebensmittelinstitut Braunschweig controls compliance with these maximum levels.

Materials and Methods: In 2007 and 2008, aflatoxin contents were determined in approx. 470 and 390 samples respectively. Sample preparation included extraction of aflatoxins with methanol and cleanup of the extracts on immunoaffinity columns. Aflatoxins were separated by HPLC and quantified by fluorescence detection after post column derivatization.

Results: Aflatoxins were detected in 34% and 28% of samples respectively. Maximum levels were exceeded in 2% of samples (mainly dry fruits and nuts) with aflatoxin B1 contents from 2.3 to 74.3 µg/kg in 2007 and from 3.4 to 38.2 µg/kg in 2008.

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SCF, Scientific Committee for Food, 1996. Opinion on aflatoxin, ochratoxin A and patulin, expressed on 23rd September 1994. *Reports of the Scientific Committee for Food*, 35th series, Luxembourg
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Occurrence of 40 mycotoxins in malt germs and other feedstuffs – determination by LC-MS/MS

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At present, only few data exist concerning a simultaneous contamination of feedstuffs with T-2/HT-2 and other mycotoxins, like deoxynivalenol (DON), zearalenone (ZEA), fumonisins, and ochratoxin A (OTA). For this reason, the EC-member states were advised in Commission Recommendation 2006/576/EC to provide more data with regard to the extent of co-occurrence of the mentioned mycotoxins. Therein, recommendations are given for DON, ZEA, OTA, and fumonisins. However, aflatoxin B_1 is the only EC-regulated mycotoxin in animal feed so far (Commission Directive 2003/100/EC).

The Bavarian Health and Food Safety Authority analysed 54 feed samples for the occurrence of 40 mycotoxins using a newly developed LC-MS/MS multi-method. Amongst others, this method included type A, B, and D trichothecenes, fumonisins, ZEA, aflatoxins, and OTA. To compensate for matrix-suppression effects, ^{13}C-stable isotope standards were applied. The different feedstuffs analysed were barley malt germs, maize grains, maize silages, oat grains, and other oat-containing feed.

For example, DON, ZEA, OTA, T-2, and HT-2 were detected simultaneously in 43 % of the malt germ samples (n=14). The mean concentration of T-2 and HT-2 (sum of the toxins) was 87 µg/kg. The values ranged between 5.6 µg/kg and 408 µg/kg. Fumonisins were not detected in any malt germ sample. DON and DON-3-glucoside occurred simultaneously with a rate of contamination of 100 %, each, and maximum values of 381 µg/kg and 414 µg/kg, respectively. The results are presented and discussed according to current regulations.

References:

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Commission Directive 2003/100/EC of 31 October 2003 amending Annex I to Directive 2002/32/EC of the European Parliament and of the Council on undesirable substances in animal feed. Off. J. Eur. Union, L285, 33-37.

Occurrence of aflatoxins in extracted cotton seed cake originating from South Africa

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Introduction: Within an Integrated European Union founded Project (BIOTRACER) under the 6th RTD Framework, 60 cotton seed cake samples were analysed. Samples originating from Benin have been collected in feed mills in South Africa. Aflatoxins have been found abundantly in cotton cake, cotton seed, cotton seed cake as well as cotton seed meal. Aflatoxin contents ranging from 24.0 to 112.3 µg/kg have been reported to be very high (Jaime-Garcia and Cotty, 2003). Maximum levels for aflatoxins in feed are, depending on the intended animal, ranging from 5 to 20 µg/kg (European Commission, 2003).

Material and Methods: Cotton seed cake samples were extracted with MeOH:Water (80:20), followed by the clean-up using immunoaffinity columns (AflaCLEAN™, LC-Tech, Dorfen, Germany). After a washing step using PBS, the samples were eluted with MeOH accompanied by backflushing. The eluates were evaporated under a stream of nitrogen and the residue was redissolved in mobile phase. The extracts were injected directly into HPLC and the determination was carried out using HPLC-FLD coupled to a Kobracell (postcolumn derivatisation).

Results: 60 cotton seed samples have been analysed for their aflatoxin contents. All samples were found positive for major aflatoxins. 42 out of 60 cotton seed samples exceeded the maximum levels for feed. Mean value, median, minimum and maximum values are presented in the following table.

	x_M (µg/kg)	median (µg/kg)	max (µg/kg)	min (µg/kg)
AFT	46	41	148	23
AFB_1	25	22	76	13

The average ratio of AFB_1 to the sum of AFB_2, AFG_1 and AFG_2 has been determined to be 1.26.

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European Commission, 2003. COMMISSION DIRECTIVE 2003/100/EC of 31 October 2003 amending Annex I to Directive 2002/32/EC of the European Parliament and of the Council on undesirable substances in animal feed. Official Journal of the European Union. L285, 33-37.

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Occurrence of mycotoxins in agricultural commodities from Europe in the year 2008

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Introduction:

Mycotoxins are different fungal toxins which can pollute the feed chain. According to the levels of contaminations, mycotoxins can lead to acute mycotoxicoses with severe intoxication or chronic effects like reduced feed intake, immune suppression or impairment of the reproductivity in the animal. A survey about the occurrence of major mycotoxins from the agricultural point of view in feedstuffs from Europe was initiated by BIOMIN GmbH as a part of its mycotoxins risk management program.

Materials and Methods:

In the year 2008 a total of 672 raw materials and finished feed samples from Europe were analysed for B-trichothecenes (deoxynivalenol and acetyldeoxynivalenol), zearalenone, A-trichothecenes (T-2 toxin and HT-2 toxin), aflatoxins, ochratoxin A and fumonisins. The majority of the samples were received from Austria (596). Analyses were performed by HPLC (high performance liquid chromatography) or ELISA (enzyme-linked immunosorbent assay). For the purpose of data analysis, non-detect levels were based on the quantification limits of the test method for each toxin: B-trichothecenes 50µg/kg (HPLC); deoxynivalenol and acetyldeoxynivalenol 250µg/kg (ELISA); zearalenone 10µg/kg (HPLC) or 40µg/kg (ELISA) respectively; A-trichothecenes 30µg/kg (HPLC); T-2 toxin and HT-2 toxin 75µg/kg (ELISA); ochratoxin A 1µg/kg (HPLC) or 2µg/kg (ELISA), respectively; aflatoxins 0.5µg/kg (HPLC) or 1µg/kg (ELISA), respectively and fumonisins 25µg/kg (HPLC) or 250µg/kg (ELISA), respectively.

Results:

Table 1 gives an overview of the occurrence of mycotoxins in this survey in which 672 raw material and finished feed samples from Europe from the year 2008 were analyzed. As samples were analysed with either HPLC or ELISA, data on “positive” samples were based on the limits of detection for ELISA analysis.

Table 1: Overview of the survey results

	Sample number	Positive [%]	Average of positive [µg/kg]	Max. Level [µg/kg]
B-trichothecenes	654	63	653	24019
Zearalenone	413	22	29.6	902
A-trichothecenes	43	7	9.6	115
Ochratoxin A	38	13	3.0	54
Aflatoxins	57	26	33.8	1621
Fumonisins	26	38	2097	13622

Discussion and Conclusions:

The occurrence of mycotoxins in samples from Europe varied from 63%, 22%, 7%, 13%, 26% and 38% for B-trichothecenes, zearalenone, A-trichothecenes, ochratoxin A, aflatoxins and fumonisins, respectively. These results indicated the necessity of monitoring mycotoxins to create an awareness of the risk of mycotoxin contamination in feedstuffs.

Appreciations of the sea buckthorn fruits (*Hippophae rhamnoides* L.) growing in the spontaneous flora of Transylvania, used as source of phyto-pharmaceutical products, by chemical and mycotoxicologic analysis

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Introduction: Sea buckthorn (*Hippophae rhamnoides L.)* grows spontaneously in Romania, especially in the river bed from hill areas. The fruits (*Fructus Hippophae*) are used fresh or dry, being harvested after maturation but before the first frost. The fruits are considered real natural poly-vitamin and "energetic bombs" offered by the nature. The harvesting, conditioning and storage influence the quality with repercussions upon all the products processed from these fruits.
The realized studies are continuing other approaches made during the last years upon the sea buckthorn fruits from the neighborhood of Cluj and Alba (Center of Transylvania) and upon the oil extracted from the fruits.

Material and Method: There were sampled a number of 14 samples from 7 different locations. After harvesting the sea buckthorn fruits were artificially dried at the temperature of 50-60°C. The samples were stored in paper bags. After 5 months, there were analyzed: the organoleptic characteristics, the raw chemical compositions (classical methodology), the oil composition (fatty acids profile by CG-chromatography), the bacteriologic charge (TGN/g) and the fungi charge (CFU/g by cultivation on solid Sabouraud medium). There were identified the most frequent fungi species on the base of the cultural and morphological characters (stereo-microscopic, on preparations slides colored with bleu cotton).

Results and Conclusions: The organoleptic examination did not ascertained modification of the aspect, color, odor/smell and taste of the samples. The results of the raw chemical composition were comparable with data form the literature. Between the samples, the differences were not significant. The TGN/g value was below 200 thousand in all samples. For 14% of the samples the fungi charge was below 0.2 millions. For the other samples the values were between 0.275 and 0.84 millions CFU/g. The dominant fungi were from two species of *Penicillium* (higher frequency for *Pencillium crustosum*). In 50 % of samples developed species exclusively from genus *Penicillium*. Based on the observations and the analysis results, the conclusion was that the contamination with mycets occurred during the period from harvesting until drying. Regarding the mycotoxic potential of the fungi from the genus *Penicillium*, there are made recommendation concerning the prophylaxis of sea buckthorn fruits contamination.

Reference:

Dancea Zoe, M. V. Morar, C. Bele, A. Macri, M. Mircean, A. Szakacs, I. Pop, E. Pall, 2006, Researches regarding the medicinal properties of the plant species Hippophae Rhamnoides L. und Adonis Vernalis L., with an increased incidence in Transylvanian spontaneous flora, Tagungsband Potenziale zeitgemäßer Qualitätsforschung - Von der Methode zum Ganzen, Universität für Bodenkultur Wien, 66-68

Distribution of trichothecenes and zearalenone in maize plants after artificial inoculation with *Fusarium graminearum* at two harvest timepoints

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We investigated the spread of fungal mycelium and the presence of mycotoxins in the stem below and above artificially inoculated maize cobs at silage maturity and grain maturity.
Twenty maize inbred lines were inoculated in a field trial with a conidia suspension containing $1x10^5$ conidia ml^{-1} of *Fusarium graminearum* five to seven days after female flowering by injection into the silk channel. The plants were scored for visual symptoms of cob infection and harvested at dough stage (46 dpi) and grain maturity stage (77 dpi). Harvested plants were separated in three parts: the infected cob and the internodes below and above the cob. The samples were analyzed for the content of deoxynivalenol, 3-acetyl-deoxynivalenol, 15-acetyl-deoxynivalenol and zearalenone with HPLC-MS/MS. *Fusarium graminearum* DNA was quantified by real-time PCR.

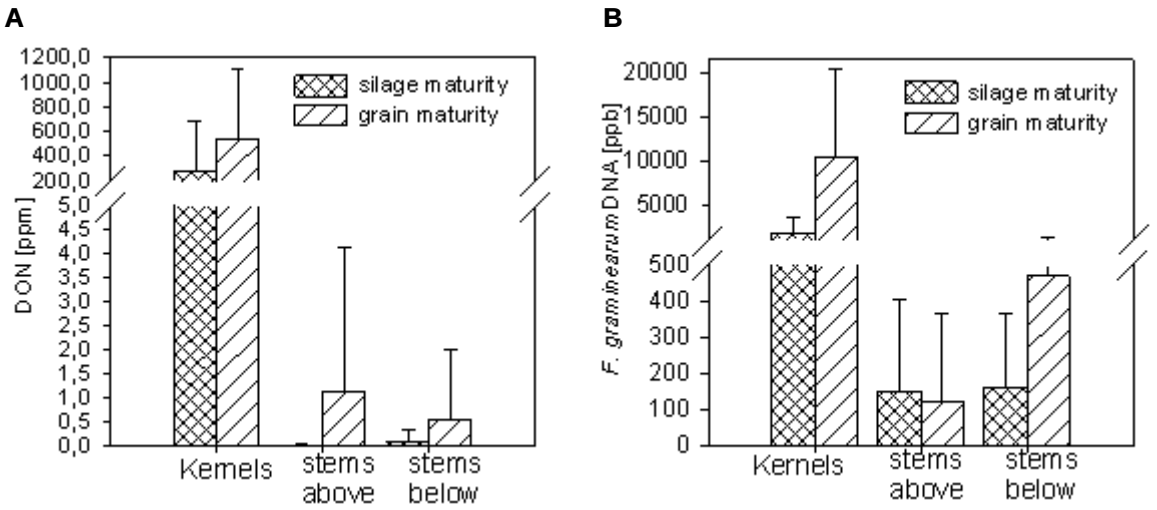

Fig.1 Distribution of deoxynivalenol (A) and *F. graminearum*-DNA (B) in the infected cob and stem above and below the cob

The highest accumulation of trichothecenes and zearalenone was detected in kernels during ripening process from dough stage to grain maturity stage. In the stems low mycotoxin contents were detected at silage harvest. At grain harvest higher mycotoxin contents could be observed in the stems above the infected cobs than below the cob. In contrast to mycotoxins, the amount of *F. graminearum* DNA did not increase in aging stems above the infected cob but the fungus grew in the stems below the cob.
Visual score of cob infection did not correlate with mycotoxin content in the stem.

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Morphology, cytotoxicity and mycotoxin production of *Stachybotrys* spp.

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The species concept of *Stachbyotrys chartarum* is not well delineated and has been subject to controversy since it was proposed as the type species of the genus *Stachybotrys* under the name of *S. atra* by Corda in 1837. Recent studies have shown that *S. chartarum sensu lato* included several closely related species and cryptic species, which raise the question concerning common identification reports of *S. chartarum* (1,2).
The objective of the work presented here was to study the possibilities of differentiation between selected strains, the problematic aspect of *S. chartarum* identification and the mycotoxin production by a highly toxic chemotype S isolate. The methods used comprise culturing of the isolates on different media, light and electron microscopy, a cell culture bioassay (MTT-assay) for cytotoxicity, HPLC analysis for the detection of satratoxins, an ELISA for particular macrocyclic trichothecenes and a PCR method for the *tri5*-gene, the key gene for trichothecene biosynthesis.
The results of HPLC and trichothecene-ELISA exhibited a good correlation with those of the MTT-cell culture test. The highly toxic macrocyclic trichothecenes satratoxin G and H were only determined chromatographically in extracts from strains which exhibit high cytotoxicity. The *tri5*-gene was determined in all toxic isolates with the aid of PCR. The allocation of all *Stachybotrys chartarum* strains to their respective subspecies was possible based on these data. Overall, three and four of seven strains under investigation were identified as *S. chartarum* chemotype and *S. chlorohalonata*, respectively.
A long-term experiment with *S. chartarum* chemotype S demonstrated a substantial toxicity already after 6 days of growth. Maximum toxicities and levels of satratoxins were measured at the beginning of the 4th week of growth. The results of this research show that a variety of feeds and construction materials are ideal sources of nutrients for the fungus *S. chartarum* chemotype S.
The following practical conclusions can be drawn:

- not every black fungus found in indoor or environmental samples belongs to the genus *Stachybotrys*,
- not every *Stachybotrys* species is a *S. chartarum*,
- not every *S. chartartum* is a chemotype S isolate with the ability to produce macrocyclic trichothecenes, and finally,
- not every isolate of *S. chartarum* chemotype S is producing toxins or has already produced under the given environmental conditions.

References:

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Revealing the first pathway intermediate of bikaverin biosynthesis in *Fusarium fujikuroi*

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The rice pathogen *Fusarium fujikuroi* is the main producer of gibberellic acids (GAs), phytohormones used in agri- and horticulture. Beside GAs, *F. fujikuroi* is able to produce the red, bioactive polyketide bikaverin, which reveales several cytotoxic effects on different human tumor and cancer cell lines. In addition, bikaverin shows an antibiotic effect against *Phytophthora infestans* and *Leishmania brasiliensis* causing light blight disease in potatoes and Leishmaniasis, respectively. Furthermore, it is an undesired by-product during GA production.

Six genes (*bik1 – bik6*) are involved in bikaverin biosynthesis. These genes are located adjacent to one another on a chromosome (gene cluster). The proteins encoded by the genes *bik1 – bik3* are most likely responsible for structure formation of the molecule, whereas Bik4 and Bik5 are likely involved in regulation of *bik* gene expression. Bik6 is expected to be a transporter responsible for the export of bikaverin or its precursors. [1]

The first bikaverin cluster gene encodes the polyketide synthase Bik1, which condenses malonyl- and acetyl-CoA units into the so far unknown first intermediate of the bikaverin pathway. This intermediate is presumably modified by Bik2, a monoxygenase, and Bik3, an *O*-methyltransferase, in order to form bikaverin. Interestingly, *F. fujikuroi* also produces nor-bikaverin, which only lacks one methoxy group at C8 compared to bikaverin. The significant accumulation of this material (a third relative to bikaverin) suggests that the methylation of the Bik3 substrate does not proceed completely.

In order to identify intermediates of the bikaverin biosynthesis, mutants deficient in one or two genes needed for structure formation of bikaverin (*bik1 – bik3)* were prepared. Unfortunately, a knock-out of one of these genes resulted in down-regulation of all other cluster genes, making it impossible to find new intermediates.

To overcome this so far unknown regulatory mechanism, the natural promoter of the *bik1* gene was substituted by the strong promoter of the *F. fujikuroi* glutamine synthetase gene, *glnA.* This over-expression of *bik1* in the double knock-out mutant ΔΔ*bik2*/*bik3* led to the first *in vivo* formation of pre-bikaverin - the product of the polyketid synthase Bik1 - which could be identified by HPLC-MS/MS. The structure was confirmed after isolation of pre-bikaverin using prep. HPLC by detailed MS- and NMR-experiments [1,2].

Using the same strategy, *bik1* was over-expressed in the Δ*bik2* and in the Δ*bik3* mutant, respectively. Unfortunately, this approach led only to the expression of the polyketide synthase gene *bik1* and a concomitant pre-bikaverin production, but not to a restored expression of the other cluster genes.

In order to identify more intermediates of the bikaverin pathway in *F. fujikuroi*, the genes *bik1* and *bik2* and accordingly *bik1* and *bik3* should be over-expressed in the ΔΔ*bik2/bik3* mutant to overcome this down-regulation-mechanism.

References:

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Mycotoxin occurrence in different genotypes of *Triticum monococcum* and *Triticum dicoccum*

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The increasing interest of organic farming and health food products has led to renew of hulled wheat species growing such as emmer (*Triticum dicoccum)* and *Triticum monococcum* (De Vita, et al 2006). Knowledge of quality traits as content of mycotoxins in these cereals is required by consumers. Mycotoxins are secondary metabolites produced by different species of fungi contaminating e.g. cereals. Most frequent mycotoxins which occur in cereals are trichothecens produced by fungi from genus *Fusarium* but also aflatoxins and ochratoxins produced by fungi from genus *Penicillium* and *Aspergillus*. The aim of this study was determination of mycotoxins in wholemeal flour in genotypes of *Triticum monococcum* and *Triticum dicoccum*. The presence of mycotoxins: aflatoxin B1 and B2, ochratoxin A, T-2 toxin and deoxynivalenol was determined by Enzyme-Linked ImmunoSorbent Assay (ELISA) method.
Among 3 of the examined cultivars of *Triticum monococcum* deoxynivalenol was identified in all samples (up to 0.31 mg/kg), T-2 toxin was identified in one sample (179.6 µg/kg), while aflatoxins and ochratoxin A were not found. Among 3 of the examined cultivars of spring *Triticum dicoccum* deoxynivalenol was identified in two samples (up to 0.3 mg/kg), T-2 toxin, aflatoxins and ochratoxin A were not found. Among 5 of the examined cultivars of winter *Triticum dicoccum* deoxynivalenol was identified in three samples (up to 0.3 mg/kg), T-2 toxin, aflatoxins and ochratoxin A were not found.

Reference:
De Vita, P.; Riefolo, C.; Codianni, P.; Cattivelli, L.; Fares, C., 2006. Agronomic and qualitative traits of *T. turgidum* ssp. *dicoccum* genotypes cultivated in Italy. Euphytica, 150, 1-2, 195-205.

An occurrence of *Fusarium* mycotoxins in transgenic Bt-maize vs. non-Bt-maize hybrids – a review

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Heavy pest infestation makes maize plants more susceptible to fungal infections. The European corn borer (ECB) (*Ostrinia nubilalis* Hübner) reportedly promotes the infection by *Fusarium* spp. Stalk and ear rots caused by *Fusarium* spp. are often related to mycotoxin accumulation in maize kernels. As a result, food and animal feed from maize is more severely contaminated with *Fusarium* mycotoxins: e.g. fumonisins (FUM), deoxynivalenol (DON) and zearalenone (ZEA) [1-3].

Bt maize (e.g. MON 810) is primary an important potential tool for insect pest protection, both in the EU and in other countries. Bt maize carrying the Bt gene (*CryIAb*) are highly resistant to ECB larval feeding according to delta toxin production.

Effective measures to combat pests therefore often have a positive side-effect in that they also reduce mycotoxin levels [1-5].

Metaanalysis was used to the confirmation of the reduction of *Fusarium* mycotoxins in Bt maize. More than thirty studies were analysed.

The currently-available varieties of Bt maize have shown strong evidence in field conditions worldwide of having significantly lower FUM levels than non-Bt isolines [2,4]. There is also limited evidence for lower levels of DON and ZEA in Bt maize, although there are fewer field studies on these relationships [4,5].

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1. Munkvold, G.P.; Hellmich, R.L.; Showers, W.B., 1997. Reduced *Fusarium* ear rot and symptomless infection in kernels of maize genetically engineered for European corn borer resistance. Phytopathology, 87, 1071–1077.
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3. Magg, T.; Melchinger, A.E.; Klein, D.; Bohn, M., 2002. Relationship between European corn borer resistance and concentration of mycotoxins produced by *Fusarium* spp. in grains of transgenic *Bt* maize hybrids, their isogenic counterparts, and commercial varieties. Plant Breed., 121, 146–154.
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Controlling in silos as a possibility to avoid mycotoxins in maize silage

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Introduction: The hygienic quality of maize silage has a great influence on the quality of food of animal origin. New safety standards require health care for animals and consumers of animal products. To reach this aim, we need a controlling for the production of silages. Therefore a comprehensive research project was conducted. The quality of grass and maize silages was studied, including the investigation of the microbiological and mycotoxicological state of the samples. Here we present some results for the maize silages.

Materials and Methods: Samples were taken from 45 farms and 5 research stations, including the measurement of temperature and physical properties of the silages. The dates of sampling were also recorded. For the analysis of quality control, the method of ensiling, temperature changes at different depths from the face of the open silo and compaction were took into account. From 118 maize silos, 304 samples were examined for microbiological and mycotoxicological conditions.

Results: The calculated filling intensity ranged from 29 to 53 t FM/h. For packing, vehicles of 5 to 15 t of weight have been used. The filling of the silos lasted between 8 to 9 hours. The silo volumes ranged from 400 to 530 m³. Measurement of temperature revealed that nearly 30 % of the silages had a temperature difference greater than 5 Kelvin, which indicates reheating. There is also a strong relationship between the density and silage losses. The mean of density increased from the top to the bottom, 219 ± 48 to 273 ± 41 kg DM/m³. With a higher DM the density increased by 6 kg DM/m³ per % point. In different sample qualities, normal (47%), reheated (49%) and moulded (77%), metabolites of *Aspergillus fumigatus* were found (Ostertag *et al.*, 2009). In moulded maize silages tremorgenic metabolites like fumitremorgin B have been detected in concentrations up to 6 µg/kg DM. To avoid reheating reduces nutrition losses of silages and cares animal health. Moulded feed should not be fed is the advice for farmers. A well balanced controlling for farmers and their partners is a prevention instrument and avoids mycotoxins in silage.

Reference:

Ostertag, J.; Bauer, J.; Spiekers, H.; Richter, W.; Meyer, K.: Secondary metabolites of *A. fumigatus* in grass and maize silages. Proc. Soc. Nutr. Physiol., 2009, 18, 61.

Financial support: The work was supported by a project of the Bavarian State Ministry for Nutrition, Agriculture and Forestry: “Hygiene of Bavarian Silages” (A/06/06).

Effects of *Fusarium* infection of wheat on mycotoxin concentration in straw when stored under different conditions

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Introduction: *Fusarium* head blight is an important plant disease in cereals. As consequence thereof not only the grains might be contaminated with mycotoxins but also the remaining parts of the plant, i.e. straw and chaff. Cereals straw is mostly used as bedding material, but also as a structural component in feeding. Straw is sometimes stored under unfavourable conditions which enable a post-harvest water uptake which favours initiation and progression of microbiological destruction and mycotoxin formation.
The aim of the present experiment was to investigate the time course of the concentration of mycotoxins of *Fusarium* infected and not infected straw under different storage conditions.

Materials and Methods: The wheat straw (Straw from *Fusarium culmorum* inoculated [F] and not inoculated wheat [C]) was stored either in a barn (B) or outdoor (O) covered by a tarp. Samples of the four variants of straw (CB, FB, CO and FO) were taken at the beginning of the storage period and after 2, 4, 8, 16 and 32 weeks. The samples were dried, ground to pass a 1 mm screen and frozen at -18°C until analyses. Zearalenone (ZON) and ochatoxin A were determined by HPLC with fluorescence detection. The trichothecenes deoxynivalenol (DON), 3-and 15-acetyldeoxynivalenol (3- and 15-AcDON), nivalenol, fusarenon X, T-2 toxin, HT-2 toxin, T-2 triol, T-2 tetraol, neosolaniol, scirpentriol, 4,15-diacetoxyscirpenol, 15-monoacetoxyscirpenol were determined by GC-MS.

Results: There was a large water uptake in the sample stored outdoor with a decrease of dry matter down (DM) to 20 percent in individual samples whereas the straw stored in the barn remained at approximately 84 percent DM. NIV was only found in a few samples and 3-AcDON only in the samples of the inoculated straw. 15-AcDON was detected in nearly all samples with slightly higher values in the inoculated straw and a small decrease over time. With maximum single values of above 40 mg/kg, DON occurred 6 to 20 times higher in the inoculated straw then in the control straw. Also the amount of ZON was higher in the inoculated straw with an increase over time in the straw stored outdoor. All other toxins were lower then the detection limits.

Conclusions: Only ZON seemed to be influenced by the different storage conditions with an increase over time which might be related to the decrease of DM. The variation for all other toxins was probably caused by pre-harvest differences.

Effect of stress factors and carbohydrates on the production of fumonisins by *Fusarium verticillioides*

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Fumonisins are a group of mycotoxins mainly produced by *Fusarium verticillioides* and *Fusarium proliferatum*. These toxins, especially Fumonisin B1, cause leucoencephalomalacia in horses and pulmonary oedema in pigs and are cancerogens for humans. The function of fumonisins and environmental conditions which lead to their production are unclear. It is known that the production of fumonisin strongly depends on pH, the availability of nitrogen and temperature. Previous work showed stimulation of fumonisin biosynthesis by certain carbohydrates. The objective of the present study was to analyse the effect of stress parameters and carbohydrates on the production of fumonisins by *Fusarium verticillioides*.

Liquid media in Erlenmeyer flasks were inoculated with spores of *Fusarium verticillioides* and amended with hydrogen peroxide, fusaric acid and different carbohydrates. Furthermore, dual cultures were investigated. In some experiments the time course of the production of fumonisins, biomass and pH-value of the medium were monitored. The cultures were filtered and mycotoxin content was determined using HPLC with mass spectrometry detection. Mycelium were freeze-dried and weighed. In case of dual cultures, DNA was extracted and species-specific real-time qPCR was used to determine the amount of fungal biomass.

Hydrogen peroxide reduced both the biomass and fumonisin production when it was added daily at the time of inoculation. This result shows that the factors controlling the production of fumonisins and deoxynivalenol are different, because Ponts *et al.* (2006) reported a stimulation of deoxynivalenol produced by *Fusarium graminearum* under these conditions. Hydrogen peroxide added 14 days after inoculation and then every 7 days did not effect the production of fumonisins nor the biomass.

In dual cultures fungal biomass and concentration of fumonisins decreased or were not affected. Competition between fungi does not seem to induce the synthesis of this group of mycotoxins. Carbohydrates strongly affected fungal growth and the production of fumonisins, the latter supposedly due to their effect on the pH of the medium.

Reference:

Ponts, N; Pinson-Gadais, L.; Verrdal-Bonnin, M.-N.; Barreau, C.; Richard-Forget, F. (2006): Accumulation of deoxynivalenol and its 15-acetylated form is significantly modulated by oxidative stress in liquid cultures of *Fusarium graminearum*. FEMS Microb. Lett. 258: 102-107.

Effect of the growth regulator Cycocel on the deoxynivalenol contamination of wheat

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Treatments with growth regulators are common in cereal production to limit the internode elongation of the plants thus improving the resistance against lodging. Lodging is known to increase the risk of *Fusarium* infection of the ears and the contamination of grain with *Fusarium* mycotoxins (Langseth and Stabbetorp, 1996). However, reduced stem length resulting from growth regulator application shortens the distance between phytopathogenic fungi originating from the soil and the ears being susceptible for *Fusarium* infection, even if lodging is avoided.

An experimental field study was carried out at the JKI, Braunschweig, to investigate the effect of the growth regulator Cycocel on the contamination of winter wheat grain with the *Fusarium* mycotoxin deoxynivalenol (DON). In the years of 2006 and 2007, six varieties of winter wheat differing in plant length character (rating system of the Bundessortenamt: notes 2–7) and in susceptibility against *Fusarium* head blight (rating system of the Bundessortenamt: notes 2-5) were cultivated on two main plots being ploughed or mulched with maize residues to promote *Fusarium* infection of the ears. Within these main plots the plants were either treated with 1.5 l ha^{-1} Cycocel 720 applied in two rates at the growth stages of tillering (0.6 l ha^{-1}) and 3-node stadium (0.9 l ha^{-1}) or remained untreated as control. At the stage of flowering, plant length defined as distance from soil surface to the top of the ear was determined at 20 randomized breakpoints per variant. Mature grain of each variant was harvested with a combine harvester, gently cleaned and ground to pass through a 1-mm sieve before being analysed for DON concentration with the ELISA test kit Ridascreen DON (product No. R 5906, r-Biopharm, Darmstadt, Germany; limit of quantification: 37 µg DON kg^{-1}).

In both experimental years, no lodging was observed in any plot of the field trial.

Plant length of control plants ranged between 82 and 111 cm (2006) and 72 and 114 cm (2007), respectively, whereas treatment with Cycocel resulted in plant length reduction of about 11 cm (2006) and 8.5 cm (2007) in the mean.

DON concentrations in grains varied at low levels in 2006 (≤200 µg kg DM^{-1} in ploughed plots; ≤300 µg kg DM^{-1} in mulched plots), but at higher levels in 2007 (≤870 µg kg DM^{-1} in ploughed plots; ≤1200 µg kg DM^{-1} in mulched plots).

In this study, no definite correlation between plant length and DON concentration in the grain could be demonstrated. In general, the application of Cycocel had no relevance on the level of DON contamination of the grain. Increased levels of DON were only detected in grain deriving from mulched plots compared with ploughed plots and from varieties showing a higher susceptibility against *Fusarium* head blight than varieties exhibiting good resistance properties.

Reference:

Langseth, W.; Stabbetorp, H., 1996. The effect of lodging and time of harvest on deoxynivalenol contamination in barley and oats. Journal of Phytopathology, 144, 241-245.

Evaluation of factors contributing to deoxynivalenol contamination of cereals in Czech Republic

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A survey of Fusarium mycotoxins in cereals intended for human consumption was carried out in Czech Republic for nine-year period (2000 – 2008). Wheat and barley samples were collected directly from farmers so as to represent most of regions in Czech Republic. Immunochemical method (ELISA) was used for analyses.
537 samples of wheat and 581 samples of barley collected during eight years of DON monitoring were used for evaluation of factors that may influence final level of DON. Each sample has several attributes as a temperature and precipitation during the anthesis period, precrop, variety and region.
According to studies dealing with DON prediction (Schaafsma 2007, Sip 2006) most significant role in variability plays the year, which accounted for 48% of the variation in DON levels, followed by weather condition, i. e. precipitation and temperature during the anthesis, which accounted for 34%. Maize as a preceding crop caused significantly higher DON contamination of grain than any other precrop.

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Schaafsma A. W., Hooker D. C., 2007. Climatic models to predict occurrence of Fusarium toxins in wheat and maize. Int. J. Food Micr., 119, 116-125
Sip V., Chrpova J., Sykorova S., Remesova J., 2006. Risk assessment for managing Fusarium head blight of wheat in the conditions of Czech Republic. European Fusarium Seminar, The Netherlands (19 – 22 September 2006), p. 152

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In vitro studies to control *Fusarium* sp. and reduction of mycotoxins by fungicides

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Fusarium ear blight (*Fusarium* sp.) is seen as an increasing problem in many parts of Europe. The disease is of major concern due to production of several mycotoxins by the fungi involved which pose a threat to the health of both humans and animals.
It is a disease which is highly linked to crop rotation and tillage methods. The risk is particularly high in regions where maize is a widely crop in the rotation and reduced or minimum tillage is practiced. In seasons with high rainfall levels during flowering combined with high risk situations specific fungicide programmes need to be applied during flowering.

The objective of the investigations was it to screen different active ingredients for inhibiting the mycelium growth and the mycotoxin production in medium. As pathogens *Fusarium graminearum*, *Fusarium culmorum* and *Fusarium langsethiae* were used. In the trials the efficacy of prothioconazole, tebuconazole and the mixture of prothioconazole and tebuconazole were evaluated.

For controlling *Fusarium graminearum* and *Fusarium culmorum* tebuconazole with 1.0 ppm showed efficacy of 80% after 3 dpi. The very slow uptake of prothioconazole achieved only a reduction of mycelium growth of 50%. Very low concentration of 0.01 and 0.1 ppm did not inhibit the mycelium growth; it promotes more the fungus development.

The highest efficacy in controlling *Fusarium langsethiae* was assessed by a concentration of 1.0 ppm for mixture of tebuconazole and prothiconazole.

Analyzing the mycotoxin content in medium tebuconazole reduced DON more 70% and T2/HT-2 slightly by 60%. Using concentrations of 0.01 or 0.1 ppm according to the mycelium growth an increase of DON for *Fusarium culmorum* / *Fusarium graminearum* was detectable.

Reduction of fusarium head blight infection in wheat by white-rot fungi

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One reason for contamination of cereals with mycotoxins is the crop rotation 'wheat after maize', especially when non-tillage systems are applied to protect soil from erosion. Maize stover containing fusarium spores is left on the field surface, represention an inoculum potential, infecting wheat grown in the following year. White-rot fungi may help to rapidly degradate maize stover and thus to reduce fusarium head blight in wheat.
A field trial was carried out in southwest Germany at the research station of the University Hohenheim 'Ihinger Hof' (48.46°N, 8.56°E, average yearly precipitation 690 mm, average yearly temperature 7.9°C). After the cultivation of maize cv. Shorty the stover was left on the field and wheat cv. Ritmo was grown. The experimental factors were fungi species (*Cyathus striatus 1*, *Phanerochaete chrysosporium*), tillage (tillage, reduced tillage, no tillage), time of white-rot fungi inoculum spreading (autumn, spring) and amount of white-rot fungi inoculum (150 gramm/plot; 300 gramm/plot). The trial was set up as a completely randomized block design with three replications. From wheat flowering till harvest fusarium head blight was rated visually every week on 150 ears per plot. After harvest, concentrations of Deoxynivalenol (DON) in grain were measured by ELISA.

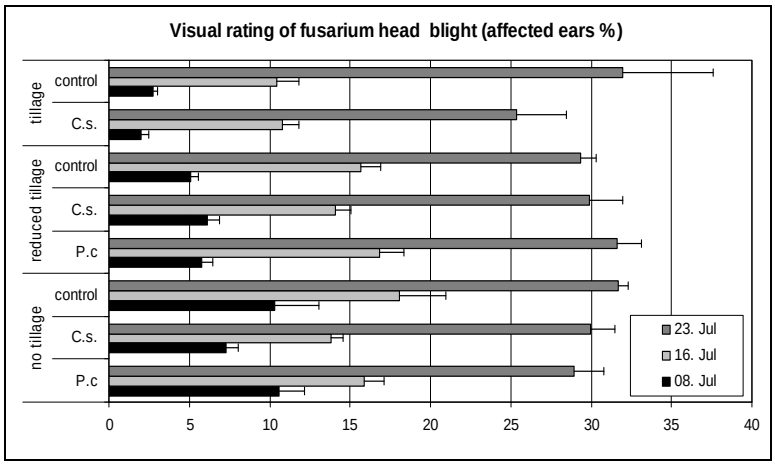

Figure 1: Visual rating of fusarium head blight in wheat (%), C.s.=*Cyatus striatus 1*, P.c.=*Phanerochaete chrysosporium*

Visual rating showed significant differences between tested tillage systems primarily in the first two ratings. As expected, the amount of infected wheat was significantly lower under tillage and reduced tillage than under no tillage. In the second rating fusarium head blight was significantly lower only in the tillage treatment. In the final rating there were no significant differences between all tillage systems. Inoculation with white-rot fungi showed no clear effects. There was no difference between time of inoculation and amount of inoculum. In the non–tillage treatment *Cyathus striatus 1* showed a trend for reduction of fusarium head blight in wheat over all rating times, but this trend was not significant. The pending DON analyses will give clearer information about the degradation potential of white-rot fungi.

Screening of the ACBR collection for trichothecin degrading yeasts

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Trichothecenes are a highly diverse group of fungal toxins acting as inhibitors of eukaryotic protein biosynthesis. Our goal is to identify yeasts with the ability to detoxify trichothecenes by irreversibly altering the toxin structure. Previously the following mechanisms of trichothecene resistance have been identified utilizing laboratory strains of *Saccharomyces cerevisiae*: 1) drug efflux, mediated by *PDR5*-like ABC transporter proteins located in the plasma membrane, 2) detoxification by formation of glucose conjugates upon expression of plant UDP-glucosyltransferases, and 3) modification of the ribosomal target (amino-acid changes in the ribosomal protein L3, product of *RPL3*).
Our working hypothesis is that yeasts may exist which have evolved the ability to detoxify trichothecenes produced by competing fungi in novel ways. Since the most relevant trichothecene mycotoxin deoxynivalenol has very low toxicity towards wild-type yeast, we utilized trichothecin in our screen. This potent toxin is produced by *Trichothecium roseum*, a fungus causing rot of grapes and other plant diseases. In one approach we have directly selected yeasts from environmental samples on YPD-medium containing trichothecin and four antibiotics to prevent growth of bacteria. A trichothecin concentration was chosen for selection that allowed growth of a *S. cerevisiae* strain with a trichodermin resistance mutation (*RPL3*^{W255C}, formerly designated *tcm1*), but blocked growth of the wild-type *S. cerevisiae* strain and most filamentous fungi. In addition we initiated a systematic screen of the BOKU yeast collection (ABCR - Austrian Center of Biological Resources and Applied Mycology, www.acbr-database.at; total 3984 accessions of ascomycetous and basidiomycetous yeasts). The yeast strains were grown in 96 well microtiter plates and replica plated to YPD plates containing increasing amounts of trichothecin. Trichothecin resistant candidates were then tested in a secondary screen for the ability to reduce the toxicity of trichothecin present in the spent culture medium. For this test 5 µl of medium was spotted on a lawn of an engineered hypersensitive yeast strain (YZAS107, relevant genotype: *pdr5 pdr10 pdr15 ayt1 ubi4 ubp6*; Abolmaali *et al.*, 2008). Candidates showing a clear reduction of the halo size should have resistance mechanisms other than drug efflux or target insensitivity. The candidates of the secondary screen are now analyzed for chemical changes of the toxins structure by HPLC-MS, which should allow distinguishing strains resistant to trichothecin due to the ability to hydrolyze trichothecin into less toxic trichothecolone from other, more interesting mutants.
Our hope is to be able to clone a detoxification gene from a promising candidate by heterologous expression of cDNAs of this organism in a toxin sensitive bakers yeast strain.

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Studies on chemical detoxification of zearalenone and deoxynivalenol

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The *Fusarium* mycotoxins deoxynivalenol (DON) and zearalenone (ZON) are of great importance because they can affect the health and performance of farm animals. In previous experiments, it was demonstrated that a treatment of wheat or triticale grains with $Na_2S_2O_5$ (sodium metabisulphite, SBS) can reduce the DON contamination nearly completely. The detoxifying effect could be confirmed by an *in vivo* experiment with piglets (Dänicke et al., 2008). Unfortunately, SBS treatment showed no effect on the reduction of the ZON contamination. In the present study, it was investigated whether other chemicals or combinations of other chemicals with SBS are suitable to decontaminate both toxins at the same time.

In a first preliminary experiment, aqueous solutions containing seventeen chemicals and combinations of chemicals, respectively (SBS, monomethyl amine (MMA), several amino acids, urea, ethylene glycol, formaldehyde and calcium hydroxide; $3.15x10^{-2}$ mM/ml each) were tested with 1 µg/ml ($3.1x10^{-6}$ mM/ml) zearalenone, in part also with 10 µg/ml DON. The solutions were shaken overnight at 50°C and ZON and DON were analyzed with HPLC/FL or HPLC/DAD
In the second experiment, 1 kg *Fusarium* contaminated triticale (80% dry matter) was mixed with 2.5-20 g/kg SBS, MMA and propionic acid, respectively, alone or in combination, and the mixtures were allowed to stand at room temperature for 18 days. ZON and DON were analyzed at the beginning and at the end of the experiment.

In the preliminary experiments in aqueous solutions, marked reduction in ZON concentration was achieved only with the mixture of MMA and SBS (reduction by more than 90%), with formaldehyde alone (reduction by more than 80%) and MMA alone (reduction by more than 50%). The mixture of MMA and SBS reduced also the DON concentration by more than 90%. Therefore, MMA, SBS and the mixture of both chemicals, partly with propionic acid as preservative, were tested in the second experiment with contaminated triticale. The best combined effect on ZON and DON showed the mixture of 5 g/kg SBS and 10 g/kg MMA (reduction of both toxins by app. 30%). Obviously, pH has a great influence on the decontamination reactions. Further studies are needed to find out whether this mixture has the potential of practical use.

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Dänicke,S.; Beineke,A.; Goyarts,T.; Valenta,H.; Beyer,M.; Humpf,H.-U., 2008. Effects of a Fusarium toxin-contaminated triticale, either untreated or treated with sodium metabisulphite ($Na_2S_2O_5$, SBS), on weaned piglets with a special focus on liver function as determined by the C-13-methacetin breath test. Arch. Anim. Nutr., 62, 263-286.

The role of zearalenone detoxification in the protection of maize against *Fusarium graminearum*

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Introduction: Zearalenone (ZEN) is a common mycotoxin produced by *Fusarium* spp. in grains. ZEN is toxic to filamentous fungi, acting as an agent of interference competition (Utermark & Karlovsky 2006). Mycoparasite *Gliocladium* is able to enzymatically detoxify ZEN. We investigated the ecological role of ZEN in the rhizosphere of maize. Five pre-screened strains of *F. graminearum* were used in combination with *G. roseum*. Maize seeds treated with fungal spores were planted into pots. Following treatments were used: Water control, *F. graminearum, F. graminearum* with *G. roseum* w.t., and *F. graminearum* with non-detoxifying mutant of *G. roseum*. Plant height, fresh shoot weight and root weight were evaluated as disease parameters. A typical result is shown in Fig. 1.

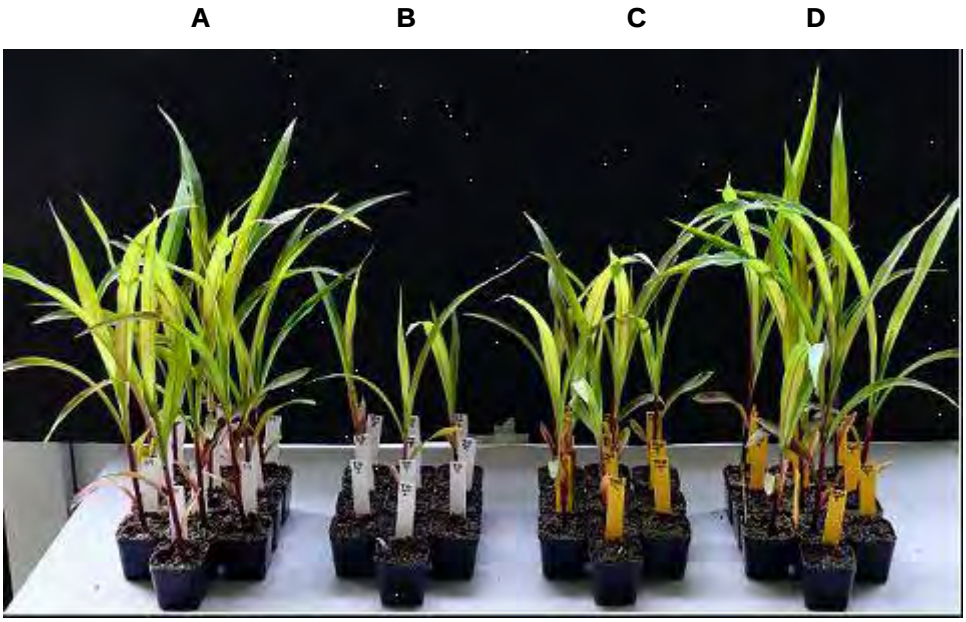

Figure 1: Effect of zearalenone detoxification on the protection of maize from F. graminearum infection by G. roseum (A) Water treated (B) F. graminearum only (C) F. graminearum + G. roseum non-detoxifying mutant (D) F. graminearum + G. roseum w.t.

Reference:

Utermark, J.; Karlovsky, P. (2007): Role of zearalenone lactonase in protection of *Gliocladium roseum* from fungitoxic effects of the mycotoxin zearalenone. Appl. Env. Microbiol. 73:637-642.

DON and HT-2 differentially affect chemokine expression in various human cell-lines.

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Introduction: Trichothecenes can be classified into groups A-D. Toxic potency, measured by *in-vitro* cytotoxicity tests, differs considerably between HT-2 (Type A) and DON (Typ B). *In-vivo*, dietary exposure to trichothecenes causes immune dysregulation that may be stimulatory or suppressive depending on exposure levels [1]. Low-doses of trichothecenes up-regulate many immune related genes e. g. those coding for chemokines [2]. The aim of the present study was to determine differences in chemokine expression of various human cell-lines after exposure to low equitoxic doses of DON or HT-2 with or without pre-stimulation of cells with LPS, a well known inducer of chemokines.

Materials and Methods: We selected some cell-lines allocated to the immune system (Jurkat T-cells, RPMI-8226, U937) as well as cell-lines that are dedicated to the initial contact with trichothecenes in case of exposure by ingestion or inhalation (Caco-2, A549). Cells were pre-incubated for 2 hours with or without 100 ng/mL LPS, followed by 3 hours of treatment with 300 ng/mL DON or 10 ng/mL HT-2 under continuous LPS stimulation. RNA was extracted, reverse transcribed and subjected to real-time PCR. IL-8 production was determined by ELISA. Experimental conditions were as follows: 20–2,560 ng/mL DON or 0.625–80 ng/mL HT-2 for 6 and 24 hours with or without LPS pre-stimulation.

Results: RNA levels of IL-8 like CXC-chemokines (CXCL1, CXCL2, CXCL8) were increased by DON in U937, Caco-2 and A549 cells, while HT-2 was only effective in A549. Presence of LPS was necessary for up-regulation of CXCL10 and some CC-chemokine transcription in all cell lines. Simultaneous exposure to trichothecenes and LPS partly potentiated effects of single substances. Overall, chemokines were least induced in Caco-2 and Jurkat cells, an exclusively LPS-dependent expression was observed in RPMI-8226 cells.

Discussion and Conclusion: Expression patterns of chemokines are different for type A and type B trichothecenes on both RNA and protein level. For immunocompetent cells, only U937 showed trichothecene-related up-regulation of chemokine transcription. IL-8 RNA expression was increased in epithelial cell-lines after DON/HT-2 treatment, whereas IL-8 protein was solely found in A549.

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DNA damaging properties of complex *Alternaria alternata* extracts in HT29 cells

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Fungi of the genus *Alternaria* produce mycotoxins such as alternariol (AOH), alternariol monomethylether (AME), altenuene (ALT) and tenuazonic acid (TA) which have been described as genotoxic and mutagenic *in vitro* and *in vivo*. Due to their ubiquitous occurrence and their potential to produce considerable amounts of toxins even at low temperature conditions, *Alternaria* spp. are associated with spoilage of refrigerated foods and stored feeds. The most prominent *Alternaria* toxins show relatively low acute toxicity. However rather little is known about the genotoxic potential and the mechanism of action of complex fungal extracts.
The aim of our present study was to establish and optimise culture conditions for different strains of *Alternaria alternata,* thus obtaining extracts with defined, reproducible mycotoxin profiles. DNA damaging effects of these extracts were investigated in cultivated human colon carcinoma cells using single cell gel electrophoresis (comet assay).

The strains *A. alternata* DSM 62010 and *A. alternata* DSM 12633 were cultivated on moist rice at 25°C in the dark. Subsequently rice samples were extracted with acidified water/ethyl acetate and the levels of AOH, AME, ALT and TA were analysed by HPLC/DAD/UV. Genotoxic effects were detected in HT29 cells after 1 h of incubation. Thereby a modification of the comet assay was implemented using the repair enzyme formamidopyrimidine DNA glycosylase (fpg) from *E. coli* in order to detect fpg-sensitive sites indicative for oxidative DNA damage. To identify potential genotoxic components of the fungal extracts, toxicity guided fractionation was performed by collecting the eluate at the outlet of the HPLC-detector. Both *A. alternata* extracts exhibited potent genotoxic properties. The increase in DNA strand breaks significantly exceeded the effect of the single mycotoxin AOH or AME at respective concentrations. Moreover, treatment with fpg significantly enhanced the DNA strand breaking properties of the extracts, whereas AOH or AME caused no fpg-sensitive sites in HT29 cells. Oxidative DNA damage was mainly induced by three peak fractions of the HPLC chromatogram, comprising components yet to be identified.

In summary, the genotoxic effects observed with complex extracts of *A. alternata* DSM 12633 and *A. alternata* DSM 62010 are not exclusively mirrored by respective amounts of AOH or AME. Furthermore complex extracts as well as fractions identified by toxicity guided fractionation led to the induction of fpg-sensitive sites in the DNA, which is not observed with the single mycotoxins AOH or AME. Thus further, yet to be identified mycotoxin(s) contribute substantially to the DNA damaging effects of *Alternaria* extracts.

Effects of feeding diets containing practically relevant concentrations of DON on the expression of inflammation related genes in pigs

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The immunomodulatory properties of deoxynivalenol (DON) have been extensively studied in various cell cultures and laboratory animals. Mechanistic studies hypothesised that DON induces phosphorylation of mitogen activated protein kinases which in turn leads to expression of inflammation related genes like tumour necrosis factor (TNF)-alpha and interleukine (IL)-6. However, in pigs, which react most sensitive to exposure to DON with a decreased feed intake as the most prominent symptom, mechanistic studies are scarce. Taking the anorectic properties of proinflammatory cytokines into account, an involvement of the proposed modes of actions seems to be possible in the reduction of feed intake observed pigs. Therefore the present investigation aimed to investigate the effects of feeding diets containing DON contaminated wheat to pigs on the expression of inflammation related genes in relevant organs.

Methods: The mRNA expression of TNF-alpha, IL-6, IL-10 and inducible nitric oxide synthase (iNOS) were analysed in samples of 30 pigs derived from a growth experiment (Döll *et al.*, 2007). Pigs were fed diets containing 0, 25 or 50% contaminated wheat (CW, 2.5mg DON/kg) as mash or pellets. After 4 weeks of feeding blood was taken from 20 pigs (diets containing 0 or 50% CW) and peripheral blood mononuclear cells (PBMC) were isolated. After 11 weeks pigs were slaughtered and samples of peyer´s patches, spleen and liver were collected. The mRNA expression of the mentioned genes was analysed by quantitative RT-PCR.

Results: The diet form (mash or pellet) did neither affect any of the investigated parameters nor interacted with the contaminated wheat.
Feeding contaminated wheat significantly increased the relative expression of TNF-alpha and IL-10 in PBMCs while the expression of IL-6 and iNOS were not affected. At the time of slaughter the expression of TNF-alpha tended to be decreased in peyer´s patches ($p=0.072$) and spleen ($p=0.113$). The relative mRNA expression of IL-10 was significantly increased in peyer´s patches and in tendency increased in spleen ($p=0.091$) by feeding diets containing increasing proportions of contaminated wheat. The relative expression of IL-6 and iNOS were not affected in any of the investigated tissues.

Conclusion: The present investigations showed that feeding DON contaminated diets induces immunomodulatory effects in PBMCs, payers patches and by tendency in spleen of pigs fed diets containing DON-contaminated wheat. Possible consequences of this immunomodulation on e.g. feed intake and feed efficacy need further investigation.

Reference:
Döll, S., Goyarts, T., Tiemann, U., Dänicke, S., 2007. Practically relevant concentrations of deoxynivalenol in diets for growing-finishing pigs offered as mash or pellets. Archives of Animal Nutrition 61, 247-265.

Impact of alternariol and alternariol monomethyl ether on Nrf2 translocation in human tumor cells

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Introduction: The mycotoxins alternariol (AOH) and alternariol monomethyl ether (AME) are secondary metabolites of the genus *Alternaria*. Because of its ubiquitous occurrence the mould *Alternaria* can contaminate food and feed thus representing a hazard to human health. AOH and AME were shown to possess genotoxic and mutagenic properties (Fehr *et al*., 2009; Brugger *et al.*, 2006; Liu *et al.*, 1991). Recently, we have reported that AOH acts as a topoisomerase poison, with preference to the II alpha isoform inducing DNA strand breaks in human colon tumor cells (Fehr *et al.*, 2009).
In this study we focussed on the role of oxidative stress and its involvement in the genotoxic properties of AOH and AME. Both mycotoxins induce the generation of oxidative species within the cells and as a consequence, the increase of cellular ROS can lead to the activation of the Nrf2 / ARE pathway. The transcription factor Nrf2 (erythroid 2p45 (NF-E2)-related factor-2) separates from its cytosolic binding partner Keap 1 (kelch-like ECH-associated protein 1), translocates into the nucleus and binds as a heterodimer to the antioxidative responsive element (ARE) inducing the transcription of metabolic phase II enzymes.
The question whether AOH and AME induce translocation of Nrf2 into the nucleus was investigated in HT29 cells by Western blot analysis. Our results show that both AOH and AME increase the amount of Nrf2 in the nuclear fraction in a concentration dependent manner. Significant effects are observed at low micromolar concentrations (≥ 0,1 µM) whereas AOH shows stronger inducing properties than AME. Furthermore the incubation time plays a decisive role. After treatment with AOH and AME for 20 min the Nrf2 level in the nucleus remains unaltered. Whereas after an incubation time of 1 h and 3 h respectively, the Nrf2 concentration in the nuclear fraction is significantly increased depending on the concentration of AOH and AME.
In summary, the *Alternaria* toxins AOH and AME induce oxidative stress in human colon carcinoma cells potentially contributing to their genotoxic properties. Their impact on the Nrf2 / ARE pathway indicates a potential role of this antioxidative protection system in the detoxification of these mycotoxins.

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Impact of oxidative stress on alternariol- and alternariol monomethyl ether-induced toxicity 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. Exposure to extracts of *Alternaria* spp. has been associated with enhanced incidence of oesophageal cancer (Liu *et al.*, 1991). Genotoxic and mutagenic properties of *Alternaria* toxins such as alternariol (AOH) and AOH monomethyl ether (AME) have been described *in vitro*. However the mechanism of action has not been fully elucidated yet. Recently, we have identified AOH, a main metabolite of *Alternaria,* as a topoisomerase poison, with preference to the II alpha isoform. Topoisomerase poisoning and DNA strand breakage occurred within the same concentration range, thus topoisomerase poisoning might at least contribute to the genotoxic properties of AOH (Fehr *et al.*, 2009). The aim of the present study was to compare the toxic properties of AOH and AME in cells of different origin: colon (HT29), oesophagus (KYSE510) and liver (HepG2) thus indicating potential organ specificity. In focus was the impact of both mycotoxins on cell viability, DNA integrity and on cellular oxidative stress. Furthermore the potential induction of xenobiotic phase I metabolism associated cytochrome P450 (CYP)-monooxygenase 1A1 was investigated. Cell viability was determined after 72 h of incubation using the sulforhodamine B assay. AOH and AME significantly inhibited the growth of all cell lines tested. HepG2 cells appeared to be less potently influenced in their proliferation by AOH and AME than cells of colon/oesophageal origin. Both *Alternaria* toxins significantly increased the rate of DNA damage after 1 h of incubation at concentrations ≥ 1 µM in all tested cell lines, as measured by alkaline single cell gel electrophoresis (comet assay), indicating substantial genotoxic potential independent of organ origin. AOH and AME induced the formation of intracellular ROS in cells of colon/oesophageal origin as measured by dichlorofluorescein (DCF) assay, potentially contributing to their genotoxic properties. In HepG2 cells ROS generation was detected only after AOH incubation yet to a minor degree compared to colon and oesophageal cells. Furthermore the transcription as well as the activity of CYP 1A1 in HepG2 and KYSE510 cells was induced by AOH and AME. However, no effect on Cyp1A1 was observed in HT29 cells.
In summary, the mycotoxins AOH and AME show similar toxic properties in cells of different origin. In all three cell lines tested AOH induces the formation of oxygen species, which might at least contribute to its genotoxic properties.

References:

Fehr, M.; Pahlke, G.; Fritz, J.; Christensen, M., O.; Boege, F.; Altemöller, M.; Podlech, J.; Marko, D., 2009. Alternariol acts as a topoisomerase poison, preferentially affecting the II alpha isoform. Mol. Nutr. Food Res. ahead of print.
Liu, G., T.; Qian, Y., Z.; Zhang, P.; Dong, Z., M.; Shi, Z., Y.; Zhen, Y, Z.; Miao, J.; Xu, Y., M., 1991. Relationships between *Alternaria alternata* and oesophageal cancer. IARC scientific publications., 105, 258-62.

Inhibitory and activating effects of deoxynivalenol on the MAPK signalling pathway

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Introduction: The trichothecene DON is supposed to disrupt normal cell function by inhibition of protein synthesis [1] via binding to the ribosome and by activating cellular kinases involved in signal transduction. However, the cellular mode of action underlying these effects is not yet fully understood. Little is known about cell type-specific toxic mechanisms especially of the mitogen activated protein kinases (MAPKs) pathway and alteration of downstream transcription factor expression in “non-immune cells”.

Materials and Methods: Deoxynivalenol was purchased from Sigma Chemical Company. MAPK inhibitors (PD98059, SB203580, SP600125) were purchased from Calbiochem. Hep-G2 cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum. Apoptotic cells were quantitated by fluorescence and Western blotting using antibodies against activated caspase-3/HSP27. Phosphorylation of MAPKs and downstream transcription factors (JUN, ATF3) were assessed by Western blotting.

Results: Following 3 h to 24 h incubation, DON (1-10 µM) markedly induced phosphorylation of p38, JNK 1/2, and ERK2 in Hep-G2 cells as well as production of transcription factors JUN and ATF3. Treatment with high DON concentrations resulted in induction of caspase-3. Addition of SB203580, a specific inhibitor of p38, did not influence this reaction pattern while the MEK1 inhibitor PD98059 which blocks ERK activation had an inhibitory effect on the expression of DON-induced transcription factors.

Discussion and Conclusion: Taken together, these data suggest that DON effects on caspase-3 and transcription factors were differentially regulated and underline the role of MAPKs that coordinate with each other to control the magnitude and duration of transcription factor activity in response to DON.

Reference:

[1] Ueno, Y., 1984. Toxicological features of t-2 toxin and related trichothecenes. Fundam. Appl. Toxicol., 4, S124-S132.

Susceptibility of various human cell lines to trichothecenes

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Introduction: The *Fusarium* mycotoxins are natural contaminants in foods and occur regularly in worldwide food supplies due to mould infestation of susceptible agricultural products. Numerous studies have been conducted on the cytotoxicity of trichothecenes on a vast variety of animal and human cell lines and remarkable differences between studies using identical cell lines have been reported [1]. Therefore, the aim of this study was to generate comparative data, obtained under standardized conditions, on the cytotoxic effects of different trichothecenes on human cell lines.

Materials and Methods: Cytotoxicity of type A (T-2 and HT-2 toxin), B (deoxynivalenol and nivalenol) and D (satratoxin G and H) trichothecenes was determined by using eight permanent human cell lines (Hep-G2, A549, CaCo-2, HEp-2, A204, U937, RPMI 8226, and Jurkat). Viability of cells was measured by WST-1 assay assessing mitochondrial metabolic activity and was expressed as the concentration (IC_{50}) inhibiting 50 % of cell viability.

Results: Depending on the chemotype of the tested trichothecene, toxicity differed by a factor of 100 to 1000, the corresponding IC_{50} values were in the range from 2.2 nM (satratoxin H on Jurkat and U937 cells) to 4,900 nM (deoxynivalenol on HEp-2 cells) whereas the effect of each mycotoxin on different human cell lines was within remarkable close limits and much less pronounced as previously reported. IC_{50} values ranged from 4.4 nM to 10.8 nM for T-2 toxin, from 7.5 nM to 55.8 nM for HT-2 toxin, from 600 nM to 4,900 nM for deoxynivalenol, from 300 nM to 2,600 nM for nivalenol, and from 2.2 nM to 18.3 nM for the satratoxins. For the first time, toxic activity of trichothecenes on primary cell culture of human endothelial cells (HUVEC) was studied. The susceptibility was comparable to the other cell lines tested, IC_{50} ranged from 16.5 nM (T-2 toxin) to 4,500 nM (DON).

Discussion and Conclusion: From these findings it is concluded that the current focus of toxicological studies on lymphoid cell lines underestimates the potential hazard of these mycotoxins on human health.

Reference:

[1] Gutleb, A. C.; Morrison, E.; Murk, A. J., 2002. Cytotoxicity assays for mycotoxins produced by fusarium strains: A review. Environ. Toxicol. Pharmacol, 11, 309-320.

Tdp1 modulates the DNA damaging properties of the topoisomerase poison alternariol

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Alternariol (AOH), a widespread food and feed contaminating mycotoxin produced by the genus *Alternaria*, has been described recently to act as a topoisomerase poison with preference to the II alpha isoform (Fehr et al., 2009). To further underline the relevance of topoisomerase targeting for the genotoxic properties of AOH, the question was addressed whether in intact cells the stability of the intermediate topoisomerase-DNA complex is affected and to what extent human tyrosyl-DNA phosphodiesterase 1 (tdp1), an enzyme for the repair of stabilized topoisomerase-DNA intermediates, has an impact on AOH-mediated genotoxicity. AOH-induced DNA damage was analysed in HEK293 cells overexpressing a GFP chimera of tdp1 or the inactive mutant $tdp1^{H263A}$ and in cells suppressed in tdp1 expression by siRNA.

In the present study we demonstrate topoisomerase poisoning by AOH within cells using the in vivo complex of enzyme to DNA (ICE) bioassay. Alternariol monomethyl ether (AME), which structurally differs from AOH only at position 9, bearing a methoxy group instead of a hydroxyl residue, was included in our test panel. AOH and AME stabilize the topoisomerase-II-DNA-complex at concentrations ≥ 5 µM and ≥ 10 µM, respectively. A specificity of AOH to the II alpha isoform was observed in low micromolar concentrations. A stabilization of topoisomerase-I-DNA intermediates was not apparent.

The relevance of human tdp1 on AOH-induced genotoxicity was investigated using single cell gel electrophoresis (comet assay). Human HEK293 cells overexpressing human tdp1 exhibit significantly decreased DNA damage after treatment with AOH (≥ 10 µM) for 1 h in comparison to cells expressing the inactive mutant $tdp1^{H263A}$. A similar effect was observed with etoposide, a well known topoisomerase II poison. In accordance to these results, AOH at low micromolar concentrations enhanced the rate of DNA strand breaks in tdp1-suppressed HCT116 cells 48 h post-transfection in comparison to cells transfected with control siRNA. Camptothecin and etoposide as topoisomerase I and II poisons respectively caused comparable effects, underlining that tdp1 plays an important role in the repair of topoisomerase-mediated DNA damage.

In summary the present results strengthen our hypothesis that AOH acts as a topoisomerase II poison in intact cells, comprising a potential risk for health. The repair enzyme tdp1 was identified as a factor for the modulation of AOH-mediated DNA damage.

Reference:

Fehr, M.; Pahlke, G.; Fritz, J.; Christensen, M. O.; Boege, F.; Altemöller, M.; Podlech, J.; Marko, D., 2009. Alternariol acts as a topoisomerase poison, preferentially affecting the II alpha isoform. Mol. Nutr. Food Res. ahead of print

Effect of aflatoxin B1 on glucose- and carbachol-induced electrophysiological parameters in jejunum of broilers

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Introduction: Aflatoxin B1 (AfB1) is a well known mycotoxin of hepatotoxic and carcinogenic nature. Various studies have previously shown that AfB1 can adversely affect intestinal nutrient absorption in various species. Mechanisms for such an effect are however not known. Recently, it has been reported that AfB1 can induce spontaneous contractions in rat intestine and that this effect is mediated through cholinergic pathways (Gursoy et al., 2008). Present study was therefore planned to investigate if AfB1 can modulate glucose uptake or carbachol-induced Cl^- secretion in jejunal epithelia of chicken. Carbahol is known to mimic the cholinergic effects of acetylcholine, and is also known to independently increase Ca^{++} activated Cl^- secretion. Once triggered, Cl^- secretion can indirectly modulate Na^+ absorption and also the Na^+-coupled glucose absorption.

Materials and Methods: Eight, 28-day old broilers were killed with stunning and bleeding. Jejunum was then removed and placed in chilled buffer containing (mM): NaCl, 120 ; $CaCl_2$, 1.2 ; $MgCl_2$, 1.2; KCl, 5.5 ; $NaHCO_3$, 25; $NaHPO_4$, 2.4 ; NaH_2PO_4, 0.4 ; Mannitol, 20. After removal of serosal layers, segments from middle jejunum were mounted in a standard Ussing chamber. The apical and basolateral surfaces of epithelium were bathed with the aforementioned buffer at 37 ºC with continuous gassing with carbogen. AfB1 was added to the apical side at the levels of 0, 1.2, 2.5, and 3.7 μg/ml buffer. After 40 and 60 minutes of incubation with the toxin, 7 mM glucose and 100 µM carbachol was added, respectively. Short circuit current (Isc), and transmural potential difference (PD) were measured throughout the experiment. Data were assessed by using paired-samples *t* test.

Results and Discussion: Glucose-induced Isc was not affected by AfB1 treatment. However, percent increase in glucose-induced PD was 47% lower ($p<0.04$) at 3.7 μg AfB1/ml. Significant effects of 3.7 μg AfB1/ml were also found in case of carbachol-induced Isc and PD, which was 106 and 146% lower ($p<0.05$) respectively, as compared to the control. However, these differences were not significant when carbachol-induced Isc and PD values were expressed as percent change. Present data indicated that glucose uptake in jejunal epithelia of broilers can be adversely affected with exposure to AfB1. A provoking of cholinergic pathways could however not be confirmed with the levels of AfB1 used in the study. Further investigations are nonetheless needed in this direction.

Reference:

Gursoy, N.; Sarac, B.; Durmus, N.; Parlak, A.; Yildrim, S.; Kaya, T.; Bagcivan, I., 2008. Changes in spontaneous contractions of rat ileum by aflatoxin in vitro. Food Chem. Toxicol., 46, 2124-2127.

Comparison of the effect of the *Fusarium* toxin deoxynivalenol and cycloheximide as inhibitor of protein synthesis on the intestinal glucose und glutamine transport of broiler chickens

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Introduction: Some studies indicated that deoxinivalenol (DON) has negative effects on the active transport of some nutrients in the small intestine of chickens. The mechanisms involved remain unclear. It is known that this mycotoxin is a potent inhibitor of protein synthesis. We studied the effect of DON and cycloheximide, a protein synthesis inhibitor, on the intestinal transport of nutrients to study whether DON effects on nutrient transport are comparable to the effects resulting from an inhibition of protein synthesis.

Materials and Methods: Birds were fed the normal diet, which was free from mycotoxins. Intestinal segments of 8 chickens were taken from mid-jejunum; the tissue was mounted in Ussing chambers. After tissue stabilization, DON (10 ppm) or cycloheximide (1 mmol/L) was added. After 60 min 20 mmol/L of glucose or glutamine were added to the mucosal side. The transepithelial short-circuit current (Isc) and electrical tissue conductance (Gt) was recorded. Results are given as means ± SEM. Statistical differences between treatments were established by ANOVA and subsequent Duncan´s Multiple Range Test. Furthermore, a paired t-test was used between each two pairs of repetitions within the same treatment. Probability values (P) of 0.05 or less were considered significant.

Results: Addition of D-glucose or L-glutamine to the luminal side of the isolated mucosa of the jejunum increased ($p < 0.05$) the short circuit-current (Isc) compared to basal conditions in the control tissues. The Isc was not increased by the addition of glucose or glutamine after preincubation of tissues with DON or cycloheximide (Table 1). The remarkable similarity between the effects of cycloheximide and DON on nutrient uptake can be considered as consistent with their common ability to inhibit protein synthesis.

Table 1. Short-circuit current (Isc) in isolated mucosa of broilers after addition of D-glucose and glutamine without or with deoxynivalenol or cycloheximide

	Control	DON	Cycloheximide	p-value
1 - Glucose:				
Basal Isc (µA/cm^2)	5.3 ± 0.43	6.9 ± 1.39	7.11 ± 1.26	0.582
Isc after toxin (µA/cm^2)	----------	4.7 ± 0.65	5.11 ± 1.01	0.770
Isc after glucose (µA/cm^2)	9.6a ± 0.63	3.7b ± 0.73	4,17b ± 0.90	0.001
2 - Glutamine:				
Basal Isc (µA/cm^2)	6 ± 0.80	5.7 ± 0.75	5.7 ± 0.72	0.470
Isc after toxin (µA/cm^2)	----------	4.3 ± 0.56	4.6 ± 0.52	0.750
Isc after glutamine (µA/cm^2)	7.2a ± 0.48	4.22b ± 0.47	3.8b ± 0.48	0.001

Discussion and Conclusion: Our results revealed that the negative effects of DON on intestinal nutrient transport were mimicked by cycloheximide, suggesting that the inhibition of protein synthesis might be one major mechanism of DON toxicity in intestinal epithelia. Additionally, DON altered glutamine transport and by this may affect the cellular energy metabolism in the small intestine.

Glucuronidation of the Mycotoxin Zearalenone and the Growth Promoter alpha-Zearalanol by Human Microsomal and Recombinant UDP-Glucuronosyltransferases

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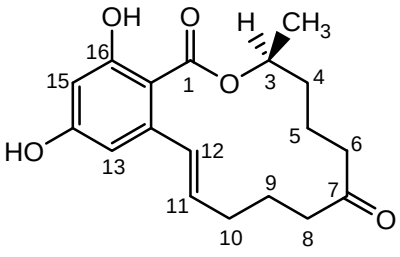
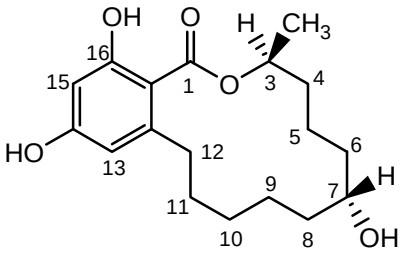
Zearalenone (ZEN) is a nonsteroidal estrogenic mycotoxin produced by Fusarium species, whereas alpha-Zearalanol (alpha-ZAL) is extensively used as an estrogenic growth promoter (Zeranol, Ralgro®) in several non-EU countries. The aim of this study was to investigate the human in vitro glucuronidation as important pathway in the phase II metabolism of ZEN and alpha-ZAL in more detail.

ZEN and alpha-ZAL were incubated with human hepatic and human intestinal microsomes in the presence of UDPGA and incubations analyzed by HPLC with UV detection. Moreover, glucuronidation of ZEN and alpha-ZAL was studied with supersomes, i.e. microsomes from transgenic insect cells expressing human UGT1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15 and 2B17.



ZEN



alpha-ZAL

When human hepatic and intestinal microsomes were incubated with ZEN, the formation of two monoglucuronides was observed, which were identified as ZEN-14-glucuronide and ZEN-16-glucuronide. The two glucuronides were generated in a 42:1 ratio by hepatic and 18:1 ratio by intestinal microsomes. In incubations of alpha-ZAL with hepatic microsomes, alpha-ZAL-14-glucuronide, alpha-ZAL-7-glucuronide and alpha-ZAL-16-glucuronide were formed in a ratio of 86:31:1; incubation with intestinal microsomes generated the three glucuronides in a ratio of 1:2:1. Incubation of ZEN with the supersomes showed that UGT1A1 was the most active isoform, followed by UGT1A8, 1A9, 1A3 and 1A7; only UGT1A6 and 2B4 were devoid of activity. Two ZEN glucuronides were formed by the active isoforms. Alpha-ZAL was also glucuronidated by numerous isoforms, with UGT1A6, 1A7, 1A10 and 2B15 being inactive. UGT1A1, 1A3, 1A8, 1A9 and 2B4 generated more alpha-ZAL-14-glucuronide than alpha-ZAL-7-glucuronide. In contrast, UGT1A4, 2B7 and 2B17 preferred the glucuronidation at position 7.

Our study has demonstrated that ZEN and alpha-ZAL are good substrates for glucuronidation by human microsomes. Although alpha-ZAL has one more hydroxyl group to glucuronidate, human microsomes have a higher activity for ZEN than for alpha-ZAL. The patterns of ZEN and alpha-ZAL glucuronides vary between the tissues and appear to depend on the tissue-specific expression of UGT isoforms. These data are of relevance for the elimination of ZEN and alpha-ZAL in humans.

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Modulation of enterocytic cell kinetics by DON

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Deoxynivalenol (DON) is a mycotoxin, frequently found in cereals used as animal feed. Chronic uptake of DON causes economical loss due to low feed intake and low weight gain in pig fattening. We have analysed the impact of high and low doses of DON on toxicity and proliferation using the intestinal porcine epithelial cell lines IPEC1 and IPEC-J2, representing the organism's first barrier for the toxin.
DON concentrations in a low (100 – 500 ng/mL) and a high (500 – 4000 ng/mL) range were applied to confluent layers of IPEC1 and IPEC-J2 for time periods of 24 h, 48 h and 72 h. Effects of DON were characterized by measuring the release of lactate dehydrogenase (LDH, cell death), tetrazoliumbromide reduction (MTT, energy metabolism) and bromdesoxyuridine uptake (BrdU, DNA synthesis).

DON exhibits distinct effects on IPEC1 and IPEC-J2 cell lines. Both were found to be insensitive to DON concentrations up to 1000 ng/mL applied for 72 h. Moderate toxic effects of DON were detected with concentrations above 1000 ng/mL and 72 h of incubation in LDH assay. Moreover, cellular viability as detected by MTT reduction was significantly reduced. In-line with these findings proliferation detected by BrdU incorporation was significantly reduced in corresponding experiments. Surprisingly, low DON concentrations between 100 and 500 ng/mL enhanced BrdU uptake without any effects in MTT and LDH assay. These findings were significant for IPEC-J2 but less pronounced for IPEC1.

The concentrations used in our in vitro system were clearly above the DON concentrations found in contaminated pig feed. Acute toxicity of DON as indicated by LDH release occurred obviously only at very high dosage. However, the enhanced proliferation of epithelial cells as found with low dosages of DON, was unexpected. This is the first time that a proliferative effect of DON on intestinal epithelial cells has been reported. It remains to be elucidated whether this effect can be observed in vivo.

Oxidative metabolism of alternariol in precision-cut rat liver slices

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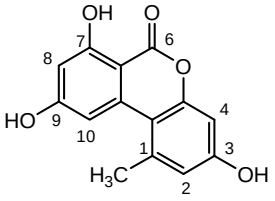
Alternariol (AOH) is a secondary metabolite of *Alternaria* fungi. The most abundant species, *Alternaria alternata*, infests numerous food items but also grows on soil, textiles and wall papers. AOH has been associated with esophageal cancer, and there are several reports on the genotoxicity and mutagenicity of AOH.



AOH

Recent studies in our laboratory have shown that AOH is extensively metabolized by rat liver microsomes. Thereby, positions 2, 4, 8 and 10 of AOH are hydroxylated (Pfeiffer *et al*., 2007. Mol. Nutr. Food. Res., 51, 307-316).

The aim of the present study was to clarify whether oxidative metabolites of AOH are formed in the presence of conjugation reactions. Therefore, we investigated the metabolism of AOH in rat liver slices, which represent an *in vivo*-like metabolic system because phase I, II and III reactions are functional.

As all four monohydroxylated metabolites are catechols, the respective monomethyl ethers were prepared from each catechol by incubation with catechol-O-methyl transferase (COMT) and S-adenosylmethionine. The catechols and their methylated metabolites then were analyzed by liquid chromatography-mass spectrometry (LC/MS).

Liver slices from male Sprague-Dawley rats were incubated with 50 µM AOH for 5 or 24 h. For the hydrolysis of conjugated metabolites, the tissue culture medium was subsequently treated with beta-glucuronidase and sulfatase, followed by extraction with ethyl acetate and analysis by LC/MS. After 5 h incubation, mainly 10-HO-AOH and 2-HO-AOH, but also small amounts of 4-HO-AOH could be detected. After 24 h, 8-HO-AOH was detectable as well. At both incubation times, methylated metabolites of all catechols except 10-HO-AOH, which is a very poor substrate of COMT, could be detected. One of the methyl ethers of 2-HO-AOH was clearly the major methylated metabolite.

Our investigations have shown that hydroxylation of AOH occurs under *in vivo*-like conditions. Therefore, the formation of oxidative AOH metabolites *in vivo* must be expected. Because of their catechol structure, these metabolites may be of toxicological relevance.

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Effects of i.v. infusion of LPS and DON on physiological and pathophysiological parameters in the pig

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Introduction: Lipopolysaccharides (LPS) are released from disintegrating gram-negative bacteria and are known to cause pronounced inflammatory responses. The mycotoxin deoxynivalenol (DON) is regarded as a potent inhibitor of protein synthesis. Moreover, the DON-related induction of pro-inflammatory cytokines was demonstrated to be boosted when primed by LPS in mice while such interactive effects for pigs were not investigated so far. Thus, the aim of the study was to test the individual and combined effects of DON and LPS on various physiological and pathophysiological parameters of the pig.

Materials and Methods: Male castrated pigs (~40 kg live weight [LW]) fed uncontaminated control feed were used for the experiments. The pigs were assigned to 4 groups and were intravenously infused with saline, DON (100µg/kg LW), LPS (7.5 to 100µg/kg LW) or LPS (7.5 to 100µg/kg LW) and DON (100µg/kg LW) for 1 h. Body temperature, heart rate and further clinical symptoms were frequently recorded up to 48 h after beginning of the infusion. Blood samples were collected for determination of haematological parameters, clinical-chemical measures and inflammatory mediators.

Results: LPS alone or in combination with DON influenced body temperature inconsistently while clinical symptoms of the induced endotoxaemia seemed to be amplified when DON was present in the systemic circulation at the same time. Blood leukocyte count was principally characterized by a temporary and pronounced LPS-dose independent leukopenia while DON did not exert an effect. LPS caused a dose dependent release of TNF-α and IL6. Moreover, this effect seemed to be even more pronounced when DON was given at the same time.

Conclusion: DON seemed to interact with LPS in different manners when various parameters were taken into consideration. Further experiments with lower systemic LPS doses, mimicking a moderate endotoxaemia in pigs exposed orally to DON are necessary to examine the relevance of the present findings.

Influence of an inoculation with mycorrhiza and toxinogenic *Fusarium* isolates on the development of winter wheat

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Fusarium head blight (FHB) is one of the most noxious cereal diseases, causing yield losses and contamination of grain by mycotoxins. The degree of FHB is influenced by many factors, especially by moisture, preceding crop and tillage. Mainly, FHB is caused by *Fusarium* (*F.*) *culmorum* and *F. graminearum*. Strains of these two species can differ in their toxicity and in their aggressiveness and might have different effects on the plant development.
Controlling and reduction of *Fusarium* infestation by pesticides, ploughing etc. is only partly successful. Thus, we need to estimate the effect of alternative methods. An important role in this context might play the mycorrhiza, a symbiotic association between plant roots and fungi. It is known that the mycorrhiza enables the plants to a higher absorption of minerals (phosphate, bonded N) and reduces the susceptibility to pathogens.

Toxicity and aggressiveness of the *F.* isolates were examined *in vit*ro. The ability to produce mycotoxins was assessed through incubation of the isolates on a rice medium. Deoxynivalenol and zearalenone were analyzed by HPLC. Aggressiveness was determined by a leaf assay involving artificial inoculation of wounded wheat leaves [1]. All tested isolates showed differences in toxicity and in aggressiveness as measured by necrotic lesion length.

In climate chamber trials we analyzed the influence of a soil inoculation with mycorrhiza and different toxinogenic *Fusarium* isolates on the development of winter wheat by analyzing the following parameters measured during ontogenesis and after harvest, respectively:

- abundance of the inoculated *Fusarium* spp. in the substrate and the whole plant
- time of formation and amount of the ears
- photosynthetic activity
- amount of chlorophyll
- degree of mycorrhization and
- activity of root enzymes (glucosidase, phosphatase).

In general, we observed that the highest differences between the inoculation varieties occurred in the beginning of ontogenesis. The mycorrhiza could compensate the negative influence of the *Fusarium* isolates. For example, during the first three months of ontogenesis the plants with mycorrhiza showed higher photosynthetic activity compared to those without.
More detailed results will be presented and discussed on the poster.

Reference:
[1] Imathiu, S. et al. (2007): *Fusarium langsethiae* pathogenicity and aggressiveness towards oat and wheat in an *in vitro* detached leaf assay. Tagungsband 29th Mycotoxin Workshop, 14th-16th May 2007, Fellbach Germany: 93.

The effects of feed naturally contaminated with mycotoxin deoxynivalenol (DON) on young breeding sows in perinatal period

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In order to establish the effects of feedstuff naturally containing mycotoxin deoxynivalenol (DON) on first pregnant sows (gilts) during the last four weeks of gestation, in the lactation period, and the period until the next insemination (54 ± 1 day), the experiment was performed on a large pig farm. Twenty pregnant gilts were divided into control and experimental group. The diet for the experimental group contained 5.08 mg DON, 0.09 mg zearalenone and 21.61 mg fusaric acid per kg. Feed for control group contained 0.29 mg DON/kg. Blood samples of gilts were obtained from *v. cava cranialis* on day 0, 17, 42 and 52 of experiment and blood samples of piglets from *v. subcutanea abdominis* 12, 24 and 48 hours after the first suckling. To obtain the colostrum samples, gilts were milked during parturition from each teat. All acquired data were statistically analyzed with the method of multiple linear regression and with t-test.

The gilts from experimental group consumed significantly less feed (P=0.026) and their piglets had a lower weight gain in the period from birth until the age of 18 days (P=0.028). Haematological and biochemical analyses of gilts' blood samples showed no statistically significant differences between groups. Special attention was paid to the indicators of immune response to DON. T helper cells ($CD3^+CD4^+$) and T cytotoxic cells ($CD3^+CD8^+$) were labelled using monoclonal antibodies (FITC anti pig CD3ε, R-PE anti pig CD4a, R-PE anti pig CD8a; BD Biosciences) and detected with flow cytometry (FACSort Becton Dickinson). The share of both subsets in experimental group was lower, reaching the statistically significant difference at day 42 of the experiment. The percentage of $CD3^+CD4^+$ and $CD3^+CD8^+$ at this time in the control group was 27.59±4.81 and 38.59±5.03 and in experimental group 23.37±3.70 (P=0.049) and 33.66±4.77 (P=0.043), respectively. Immunosuppressive effect of DON was suggested also by detection of apoptosis in the non-stimulated (P=0.036) and stimulated lymphocytes culture (1000 ng DON/ml) studied by ELISA (Cell Death Detection Elisa, Roche, 1544657). However, lymphocyte proliferation based on BrdU (BrdU Labeling and Detection Kit III; Roche, 1444611) revealed greater immunostimulating effect of DON. The peripheral blood lymphocytes were isolated and stimulated with DON (5000 and 1000 ng/ml), mitogen PHA (10 and 20 µg/ml) and by combination of PHA 10 µg/ml and DON (100, 1000 and 5000 ng/ml). Statistically significantly higher lymphocyte proliferation was found in experimental group of gilts (P<0.001), with the exception of PHA 10 µg/ml. The concentrations of immunoglobulins (Ig) were measured with the method of radial immunodiffusion in gilts' sera, their colostrums and in sera of their piglets. In colostrum samples from experimental group the concentration of IgA was statistically significantly lower (P=0.010), but in piglets' sera from experimental group there were less IgA (P<0.001) and IgG (P<0.001).

The obtained results suggest that the influences of DON on immune system of pigs are very complex and difficult to estimate.

The effect of the *Fusarium* toxin deoxynivalenol on the relative expression of SGLT-1 and GLUT-2 mRNA in intestine of broiler chickens

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Introduction: The intestinal absorption of glucose is mediated by transporters localized in the brush border membranes of the enterocytes. These transporters play a fundamental role in uptake of glucose, providing glucose as an immediate source of energy for cellular metabolism. Two types of transporters are involved in the brush border membrane hexose transport in the small intestine of higher organisms: i) facilitated glucose transporters (GLUT2) and ii) Na^+ coupled glucose transporters (SGLT-1). Quantification of expression of intestinal mRNA of SGLT-1 and GLUT2 provides important information concerning control of nutrient uptake. Therefore, in the present study, mRNA expression of SGLT-1 and GLUT2 were determined of chicken small intestine as the glucose transport is influenced by deoxynivalenol.

Materials and Methods: Total RNA extracted with a commercial extraction kit. The RNA amounts in extracts were determined with a RiboGreen RNA determination kit. A commercial mastermix was used for amplification of β-actin, GAPDH, SGLT-1 and GLUT2. The following primers were used: (β-Actin- f GAGAAATTGTGCGTGACATCA, r-CCTGAACCTCTCATTGCCA), GAPDH-f-GGTGGTGCTAAGCGTGTTA, GAPDH-r CCCTCCACAATGCCAA, SGLT1-f GCCATGGCCA-GGGCTTA, SGLT1-r CAATAACCTGATCTGTGCACCAGTA, GLUT 2-f ATTGTCCCTGGAGG-TGTTGGTG, GLUT2-r CACACTATGGGCGCATGCT). The software REST2005 was used to calculate relative expression of the SGLT-1 and GLUT2 proteins of the control group compared to the trial group.

Results: Our results revealed no significant effect of the acute in vitro DON exposure for 1 hr on both SGLT-1 and GLUT2 expression. However, from the results there is a tendency for down-regulation of GLUT2 expression in DON treated group.

Table 1. Relative expression of SGLT-1 and GLUT-2 mRNA in small intestinal (n=12)

Gene	Expression	Range	95% C.I.	p-value
GAPDH	0.980*	0.613 - 1.578	0.419 - 2.882	0.870
SGLT-1	0.979	0.484 - 2.082	0.232 - 3.590	0.900
GLUT-2	0.620	0.129 - 3.262	0.033 - 7.163	0.149

* = in relation to β-actin (GAPDH marked as target gene), β-actin is chosen as a reference gene having the same relative expression mean in the control as in the treated group =1, and the relative expression ratio for the other genes standardised by a non-regulated reference gene (β-actin), (n = number of birds, three replicates/bird).

Discussion and Conclusion: Our results revealed no significant effect of acute toxicity of DON (1 hr) on both SGLT-1 and GLUT2 mRNA expression. However, there is a tendency of down-regulation of GLUT2 expression in DON treated group. This short time of DON exposure may be not long enough for getting a clear effect on mRNA expression. However, the results from the in vitro experiments showed that DON altered activity of SLGT-1, which suggest under these experimental conditions primarily posttranslational effects of DON.

Toxicity of smut infected maize

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Introduction: Maize is an important plant grown all over the world, taking third place just after wheat and rice in the amount of crops. In some countries it may be infected with a number of toxic fungi. Surely, maize as well as other small grains cereal are often contaminated with a *Fusarium* species, however there is also a phytopathogene *Ustilago maydis* that significantly influences the number of crops, responsible for the infection called "smut".
The aim of the study was to determine the toxic and cytotoxic properties of the smut and of the *Ustilago maydis*.

Material and methods: The samples of the smut infected maize ears PR 39R10 (FAO 230/240) were collected at ZDD Swadzim from the field of 0,15ha, at the wax phase of growth (September) and full maturity (October). The controls were the maize ears not infected with the smut from the same field. Additionally, the isolates of the *Ustilago maydis* were analysed. The general number of fungi and mycotoxins content were determined. Samples, initial suspension and ten-time dilutions used for microbiological analysis were carried out according to PN–EN ISO 6887–1, July 2000. General number of moulds and yeast (cfu/g DM) was determined according to PN–ISO 7954, September 1999 (with own alternation). Cytotoxicity level was determined by the MTT test with swine kidney cells (SK), sensitive to a wide range of mycotoxins. Trichothecens and zearalenon were analysed with the HPLC-MS/MS method (3200 QTRAP, Applied Biosystem). Samples clean up was done on the Bond Elut® Mycotoxin (Varian). Ergotalkaloids were analysed with the HPLC method with fluorescent detection. While fumonisins were also examined with HPLC-MS/MS method, the samples were cleaned up on the immunoaffinity collumns FumoniTest (Vicam).

Results: High level of fungi was detected in the smut infected maize ($1,6x10^8$ and $2,5 \times 10^8$ cfu/g for the wax and full maturity) when compared to control ($6,2 \times 10^5$ cfu/g and $5,3 \times 10^4$ cfu/g for the wax and full maturity, respectively). The general number of *Ustilago maydis* detected in the smut infected maize was 3.3×10^8 cfu/g for the wax phase growth, whereas $1,7 \times 10^6$ cfu/g for the full maturity. Moreover, apart from *Ustilago* high level of *Fusarium, Acremonium* and *Penicillium* contamination was detected in the smut infected ears.
Among the trichotheces A and B, DON at the level of 236 ppb was detected in the ears not infected with the smut (full maturity), while HT-2 toxin was found in the smut infected ears (29.4 ppb). Other trichotecenes (NIV, 3-ADON, 15-ADON, DAS, T-2) and zearalenon were not detected. The samples did not contain ergotalkaloids. However, fumonisins (B_1, B_2, and B3) were found in the smut infected ears, and their level at the wax phase of growth was: B_1-387; B_2-39.5; B_3-64.1 ppb, while at full maturity it was much higher: B_1-2350; B_2-371; B_3-375 ppb. The mycotoxins levels are given at their mean value.
In the MTT test, the smut infected maize showed low cytotoxicity: IC50 50-100 mg/ml when compared to the non-infected maize: IC50 400 mg/ml. On the other hand, the MTT test of isolated strains of *Ustilago maydis* showed high cytotoxicity (IC50 0.98-0.12 cm^2/ml when compared to the control: IC50 31.25 cm^2/ml).

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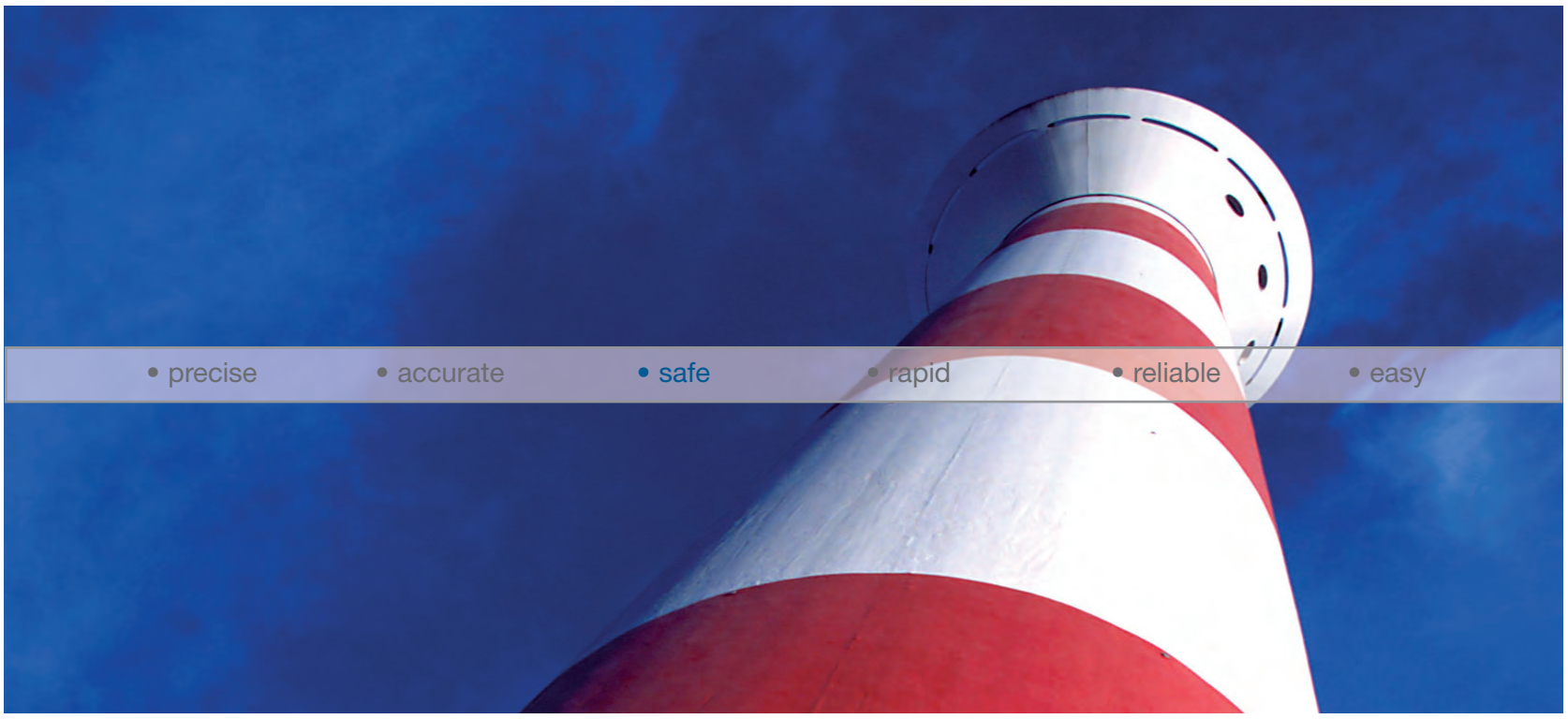

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