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Dr. I. Tothill (Cranfield University, United Kingdom)
Prof. Dr. A. Wu (SAAS Shanghai, China)

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Welcome address

Welcome to the 35th Mycotoxin Workshop organized by the Ghent University Association in collaboration with the Society for Mycotoxin Research.

Mycotoxins - toxic fungal secondary metabolites - play a significant role in food and feed safety, as well as in medical and environmental microbiology. Indeed, mycotoxins have shown to be the number one threat amongst food and feed contaminants regarding chronic toxicity. Economic losses are due to effects on livestock productivity and direct losses in crop yield and stored agricultural products. Legislative limits for a range of mycotoxins continue to develop worldwide resulting in an increased number of official controls deriving from national food safety plans and for food trade purposes. Furthermore, environmental mycotoxins are a continuous threat for human and animal health.

The challenges presented to those working in mycotoxin research are enormous due to the frequency, the complexity and variability in occurrence. Several aspects make the pre- and post-harvest control of mycotoxins difficult, such as:

- Different fungal species produce mycotoxins;
- Most of the mycotoxin producing fungi are able to produce more than one mycotoxin;
- Mycotoxin levels are influenced by environmental conditions during growth and storage;
- The presence of masked mycotoxins;
- The highly complex influence of environmental factors on the biosynthesis of mycotoxins by fungi.

Other aspects related to human and animal health also contribute to the complexity in mycotoxin research, e.g.:

- The lack of suitable biomarkers to assess exposure of humans and animals;
- The need for guidance levels of mycotoxins in animal body fluids;
- The efficacy and safety testing of mycotoxin detoxifiers;
- Knowledge about toxicokinetics in men and animals.

New developments in mycotoxin analysis focus on faster, multi-mycotoxin, environmentally friendly, cost-effective and fit-for-purpose methods in food, feed, biological tissue and body fluids. A trend towards untargeted metabolic profiling has been noticed.

Mycotoxigenic fungi, mycotoxins and food and feed safety will continue to be a critical interest to researchers for years to come. Likewise, the risks for human and animal health by indoor air contamination and other sources of environmental mycotoxin exposition are still a wide field of research. Innovations take place at a rapid pace. The investigational area is broad (phytopathology, analytical methods, risk management, toxicology, biomarkers for exposure, occupational health risks) but necessary to ensure progress into improvement of public health and animal health, in particular a safe food and feed supply.

The Mycotoxin Workshop aims to bring together experts from academia, government and industry to discuss all aspects of mycotoxin research including production, occurrence and detection, the impact on human and animal health, reduction and prevention, toxicology and other topics.

Prof. Dr. Sarah De Saeger
Prof. Dr. Siska Croubels
Prof. Dr. Hans-Ulrich Humpf
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Program

Tuesday, May 21, 2013

Meeting point: “Café het Spijker” and “Café het Gouden Mandeke”, Pensmarrkt 3 and 9, 9000 Ghent

Wednesday, May 22, 2013

8:00  Registration

8:50  Welcome
Prof. Dr. S. De Saeger, Chair Organizing Committee
Prof. Dr. S. Croubels, Chair Organizing Committee

9:00  Welcome
Prof. Dr. P. Van Cauwenberge, Rector Ghent University
Prof. Dr. G. Haesaert, Dean University College Ghent
Prof. Dr. H.U. Humpf, President of the Society for Mycotoxin Research

DETECTION
Chair: Prof. Dr. S. De Saeger and Prof. Dr. A. Wu

9:30  Combination of double isotopic labeling and high resolution mass spectrometry: a novel method for untargeted fungal metabolic profiling
P.M. Cano, E. Jamin, S. Tadrist, P. Bourdaudhui, M. Péan, L. Debrauwer, I.P. Oswald, M. Delaforge and O. Puel

9:45  Untargeted stable isotope assisted metabolic profiling reveals novel conjugates of deoxynivalenol in wheat
B. Kluger, C. Bueschl, R. Krska, M. Lemmens, G. Adam and R. Schuhmacher

10:00  Metabolic profiling of Aspergillus flavus: identification and fragmentation study of the cluster 27 polyketide synthase metabolites by high resolution and multi-stage mass spectrometry

10:15  Coffee break / Exhibition / Poster Session

11:00  LC-MS-based methods for multi-mycotoxin determination in maize: a critical evaluation
A. De Girolamo, M. Solfrizzo, V.M.T. Lattanzio, J. Stroka, A. Alldrick, H.P. van Egmond and A. Visconti

11:15  A promising grain sampling technique: the sampling of wheat dust and subsequent UPLC-MS/MS analysis
M. Sanders, M. De Boevre, F. Dumoulin, C. Detavernier, Y. Guo, F. Martens, M. Eeckhout and S. De Saeger
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<td>11:30</td>
<td>A LC-MS/MS method for the simultaneous determination of zearalenone, deoxynivalenol and metabolites in pig serum</td>
<td>U. Brezina, I. Rempe, H. Valenta, Susanne Kersten, H.-U. Humpf and S. Dänicke</td>
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<td>11:45</td>
<td>Fast and sensitive LC-MS/MS method measuring human mycotoxin exposure using biomarkers in urine</td>
<td>B. Huybrechts, P. Debongnie, S. Uhlig and A. Callebaut</td>
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<td>12:00</td>
<td>Lunch</td>
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<td>Poster Session / Exhibition</td>
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<td>13:30</td>
<td>Meeting of the members of the Society for Mycotoxin Research</td>
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<td>14:30</td>
<td>Fluorescence polarization immunoassays for the determination of mycotoxins in foodstuffs</td>
<td>V. Lippolis, M. Pascale and A. Visconti</td>
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<td>Quantum dots for use as labels for mycotoxin detection: preparation of hydrophilic quantum dots by encapsulation with polymer</td>
<td>E.S. Speranskaya, V.V. Goftman, S. De Saeger and I.Yu. Goryacheva</td>
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<td>15:00</td>
<td>Hapten microarray-based screening of mycotoxins in cereals</td>
<td>S. Oswald, R. Niessner and D. Knopp</td>
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<td>Coffee break/Exhibition/Poster Session</td>
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<td>Matrix binding of ochratoxin A during thermal processing</td>
<td>A. Bittner, B. Cramer and H.-U. Humpf</td>
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<td>16:15</td>
<td>Correlation of ergot alkaloids and sclerotia in German rye and wheat and recommendation to simplify analyses</td>
<td>E. Sciurba, S. Lewandowska, S. Seling, M.G. Lindhauer and C. Schwake-Anduschus</td>
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<td>17:00</td>
<td>Boat trip in ancient city of Ghent</td>
<td>Meeting point: Snepkaai – pier “Restaurant ‘t Patijnjte” (Gordunakaai 91, 9000 Ghent) (about 20 min walking distance)</td>
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<td>18:00</td>
<td>Welcome reception</td>
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Thursday, May 23, 2013

8:00 Registration

PRODUCTION AND OCCURRENCE
Chair: Prof. Dr. H.-U. Humpf and Prof. Dr. C. Dall’Asta

9:00 Screening biodiversity of toxigenic fungi for a correct evaluation of mycotoxin risk on important food crops: the contribution of EU project MycoRed, at a global level
A. Moretti, G. Mulè, G. Perrone, A. Susca and A. Logrieco

9:15 Fungal growth and mycotoxin production during cocultivation of phytopathogenic Fusarium and Alternaria strains
M. Müller, I. Steier, R. Köppen, D. Siegel, U. Korn and M. Koch

9:30 Interaction of free air carbon dioxide enrichment (FACE) and water availability on eoxynivalenol concentration in maize and sorghum
E. Oldenburg, R. Manderscheid, M. Erbs and H.-J. Weigel

9:45 Upregulation of sterigmatocystin and other putative mycotoxins in Aspergillus nidulans after feeding damage by Folsomia candida
M. Rohlfs, S. Chatterjee, K. Döll, P. Karlovsky and S. Scheu

10:00 Coffee break/Exhibition/Poster Session

10:45 Chlorogenic acid, a metabolite identified in tomato fruits by a metabolomics approach, is inhibitory against the biosynthesis of alternariol by Alternaria alternata

11:00 New secondary metabolites of F. fujikuroi – a combined biological and chemical approach
K. W. von Bargen, E.-M. Niehaus, B. Tudzynski and H.-U. Humpf

11:15 Tenuazonic acid in infant products – a risk for the consumer?
M. Rychlik and S. Asam

11:30 The publication of mycotoxin results exceeding the maximum levels in foodstuffs – an increase in market transparency?
S. Marschik and D. Bolay

11:45 Chemical interactions between Fusarium verticillioides and Gliocladium roseum involving mycotoxin transformation
Y. Kuang, S. Chatterjee, K. Scherlach, R. Splivallo, P. Chatterjee, C. Hertweck and P. Karlovsky

12:00 Lunch

13:00 Poster Session/Exhibition
PRODUCTION AND OCCURRENCE
Chair: Prof. Dr. S. Croubels and Prof. Dr. R. Krska

14:00  *Fusarium poae*: chemotype, plant-pathogen interaction and response to oxidative stress
L23  A. Vanheule, K. Audenaert, S. De Saeger, M. Höfte and G. Haesaert

14:15 Population structure and mycotoxin production of *Fusarium* species from greenhouse pepper fruit in China
L24  J. Wang, Z. Feng, Z. Han, S. Song and A. Wu

IMPACT ON ANIMAL AND HUMAN HEALTH
Chair: Prof. Dr. S. Croubels and Prof. Dr. R. Krska

14:30 Impact of monacolin K on animal health: preliminary test of rumen stability

14:45 Zearalenone-mediated toxicity in fish in vitro and in vivo
L26  C. Pietsch

15:00 The impact of mycotoxin co-occurrence on necrotic enteritis in broilers

15:15 Expression of immune response genes in broiler chickens fed deoxynivalenol and a counteragent
L28  K. Ghareeb, W. Awad, S. Sasgary, A. Strasser and J. Böhm

15:30 Coffee break / Exhibition / Poster Session

16:15 Detection of *Fusarium* mycotoxins and carry over to animals and humans: case study poultry
L29  E. Van Pamel, E.K. Tangni, E. Delezie, P. Debongnie, A. Callebaut and E. Daeseleire

16:30 New results for ochratoxin A analysis in infants’ urines
L30  G.H. Degen, M. Blaszkewicz and K. Munoz

19:00 Conference Dinner
Meeting point: “Handelsbeurs”, Kouter 29, 9000 Ghent
Friday, May 24, 2013

8:00      Registration

REDUCTION AND PREVENTION  
Chair: Prof. Dr. J. Fink-Gremmels and Dr. I. Oswald

9:00      Microbes as bio-control agents against *Fusarium* pathogens and mycotoxins
          L31 Y.-C. Liao, H.-P. Li, J.-B. Zhang, A.-D. Gong, W.-J. He and Q.-S. Yuan

9:15      Isolation of *Rhodococcus erythropolis* MTHt3 and cloning of ergA for ergopeptine
          hydrolysis and ergB for ergine deamination

9:30      Development of an vitro method for the prediction of mycotoxin adsorption on
          yeast-based products: case of zearalenone, ochratoxin A and aflatoxin B1
          L33 V. Faucet-Marquis, C. Joannis-Cassan, K. Hadjeba-Medjdoub, N. Ballet and A. Pfohl-Leszkowicz

9:45      Coffee break/Exhibition/Poster Session

TOXICOLOGY  
Chair: Prof. Dr. J. Fink-Gremmels and Dr. I. Oswald

10:30     New insights into mycotoxin mixtures: the toxicity of low doses of type B trichothecenes
          against intestinal epithelial cells is synergistic
          L34 I. Alassane-Kpembi, M. Kolf-Clauw, T. Gauthier, R. Abrami, F.A. Abiola, I.P. Oswald and O. Puel

10:45     New insights into the toxicological effects of altertoxin II from *Alternaria alternata*
          L35 S.C. Fleck, E. Pfeiffer and M. Metzler

11:00     Genotoxic properties of alternariol and its phase I metabolite 4-OH-alternariol
          L36 C. Tiessen, H. Gehrke, D. Ellmer, H. Mikula, J. Fröhlich and D. Marko

11:15     In silico evaluation of interactions between mycotoxins and estrogen receptors. A bio-
          informatic tool to predict and understand endocrine disrupting activity.
          L37 L. Dellafiora, C. Dall’Asta and P. Cozzini

11:30     Closing session
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<td>P4</td>
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P15 Development of rapid dipstick tests for the quantification of Fumonisins, Aflatoxins and Deoxynivalenol in grains

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M. Binder, L. Terry and I. Tothill

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J. Gerding, F. Hübner, B. Cramer and H.-U. Humpf

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P129 Deoxynivalenol affects the composition of the basement membrane proteins and influences en route the migration of CD16-positive cells
Clean up Mycotoxins with the #1 mycotoxin sequestering agent

Feeding Mycosorb, from Alltech, reduces mycotoxin absorption within the animal, thereby negating the damaging effects of mycotoxins on the health of your animals.*

Key Facts About Mycotoxins

• There are approximately 500 known mycotoxins.
• Animals are consistently exposed to multiple toxins.
• Interaction between toxins makes diagnosis difficult.
• Long term exposure to low levels of mycotoxins in the diet can reduce production and reproductive performance.
Abstracts of lectures
DETECTION - L1

Combination of double isotopic labeling and high resolution mass spectrometry: a novel method for untargeted fungal metabolic profiling

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Characterization of fungal secondary metabolomes has become a challenge of great interest in the last decades due to the emergence of fungal threats to natural ecosystems and public health; and also due to the industrial interest of many of these molecules. In view of this, the aim of the present study was to develop an integrated approach to analyze fungal metabolomes. The method we present hereby combines high resolution mass spectrometry and double isotopic labeling which efficiently enabled the unambiguous determination of exact chemical formulas, getting rid of problems coming from interference of non-biological molecules. More precisely, the *Aspergillus fumigatus* strain NRRL 35693, an extremely hazardous human pathogen and the *Fusarium graminearum* strain PH1, a devastating plant pathogen, were grown on wheat grains (*Triticum aestivum*) with different isotopic enrichments: (1) naturally enriched grains, (2) grains enriched 96.88% 13C, (3) grains enriched with 53.37% 13C and 96.8% 15N. Methanol extracts of each culture was then analyzed by reversed phase liquid chromatography coupled to LTQ-Orbitrap mass spectrometer. Data of the 3 cultures were cross-analyzed with an in-house developed software. Metabolites were characterized with the metabolite database, Antibase 2012, annotated with MS/MS experiments and identified by comparison with standards when possible. The method was firstly successfully validated with the well-known metabolome of *Aspergillus fumigatus*. Application of the method on the metabolome of *Fusarium graminearum* allowed the characterization of 37 new compounds including fusaristatin A which had never been isolated from this specie before, bringing a new perspective on the toxicity of this fungus. This kind of analysis will undoubtedly facilitate the study of fungal metabolomes.
Untargeted stable isotope assisted metabolic profiling reveals novel conjugates of deoxynivalenol in wheat

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This study will present a novel approach for the untargeted screening of metabolisation products of xenobiotics in plants using liquid chromatography – high resolution mass spectrometry (LC-HRMS). The developed methodology makes use of uniformly \( ^{13}C_{15} \) labelled deoxynivalenol (DON) and was successfully applied to wheat plants after treatment with a mixture of \( ^{13}C_{15} \)– and non-labelled DON. Two wheat lines Remus (susceptible) and CM-82036 (resistant) were challenged by injection of the prepared DON mixture in two adjacent spikelets after 0, 24, 48, 72 and 96 hours resulting in a total amount of 100 \( \mu \)g DON per ear. 108 hours after the first inoculation, treated ears were harvested, immediately frozen in liquid nitrogen and stored at -80°C until analysis. Frozen plant samples were homogenised, extracted and analysed by LC-HRMS. Automated data processing with the in house developed software MetExtract [1] resulted in the assignment of a total of nine different DON conjugates. Besides the well-known DON-3-O-glucoside, additionally DON-glutathione (GSH), DON-S-cysteinyl-glycine, DON-S-cysteine have been annotated. Moreover, several other DON conjugates were reported for the first time based on a mass deviation of less than ±5ppm and MS/MS measurements [2].

This contribution will explain the analytical approach and the complete workflow in detail. Moreover, putative molecular structures of the identified (novel) DON conjugates will be presented and the formations of these DON-biotransformation products in wheat were monitored over a time period of 96 hours after inoculation.

References
Metabolic profiling of *Aspergillus flavus*: identification and fragmentation study of the cluster 27 polyketide synthase metabolites by high resolution and multi-stage mass spectrometry

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The filamentous fungus *Aspergillus flavus* produces the toxic and carcinogenic, polyketide-derived family of secondary metabolites termed aflatoxins. In addition, in silico analysis of the *A. flavus* genome identified 54 other gene clusters predicted to be associated with secondary metabolism. In *A. flavus* cultures, approximately 20 distinctly different metabolites have been identified, however the gene clusters required for biosynthesis are only known for 3 of these. To decipher the function of some of these gene clusters, polyketide synthase (pks) mutants have been generated. Recombinational inactivation of the cluster 27 pks gene (pks27) resulted in *A. flavus* transformants that no longer produced characteristic dark brown sclerotia but instead produced sclerotia that were greyish-yellow. Extracts of the parental strain were compared to those from the Δpks27 mutant through a metabolomics approach based on ultra-high performance liquid chromatography (U-HPLC) – Orbitrap mass spectrometry. This analysis revealed, in negative electrospray ionization (ESI) as well as in negative atmospheric pressure chemical ionization (APCI), four metabolites that are present only in the parental strain. Linear Ion-Trap multi-stage mass spectrometry (MS²) and accurate mass data analysis allowed identification of the major metabolite produced by cluster 27 as asparasone A, a result confirmed by comparison to an authentic standard of this metabolite. Subsequently, the ESI- and APCI- fragmentation pattern of asparasone A was elucidated for the first time. Based on the established fragmentation pathway and mass spectral data, two other compounds, the dehydration and deacetyl derivatives of asparasone, were identified.
DETECTION – L4

LC-MS-based methods for multi-mycotoxin determination in maize: a critical evaluation

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A proficiency test (PT) was conducted within the EU Network of Excellence (MoniQA) for laboratories currently using LC-MS/(MS) for the simultaneous determination of up to 11 mycotoxins (aflatoxins B₁, B₂, G₁ and G₂, fumonisins B₁ and B₂, ochratoxin A, deoxynivalenol, T-2 and HT-2 toxins and zearalenone) at levels around the relevant EU regulatory limits. This gave participants the opportunity to benchmark their method against a rather large pool of similar methods. Most participants (61%) performed the simultaneous determination of the 11 mycotoxins, whereas the others reported results for different combinations of mycotoxins. The most frequently used extraction solvent mixtures were acetonitrile-water (acidified or not) (56%) and methanol-water mixtures (17%). Other laboratories performed two consecutive extractions with phosphate buffer solution (PBS) followed by methanol (15%) or used mixtures of acetonitrile-water-methanol (7%), water-ethyl acetate (2.5%) or PBS alone (2.5%). The majority of laboratories (58%) used a clean-up step prior to mycotoxin detection, whereas the remaining ones analysed directly the crude extract (37%) or used both approaches (5%). The amount of matrix equivalent injected into LC-MS/(MS) ranged between 0.1-303 mg for purified extracts and 0.08-20 mg for directly analysed crude extracts. External (54%), matrix-matched (22%) or stable isotope-labelled internal standards calibration (24%) were used for toxin quantification.

The evaluation of the PT results in relation to analytical parameters permitted the identification of the strengths and weaknesses of the various methodologies used by participating laboratories. In general, extraction mixtures of water with acetonitrile and/or methanol provided good results for quantitative extraction of mycotoxins from maize, however a high variability of results was observed with acetonitrile-water extraction solvent mixtures as compared to the other ones. Laboratories using sample extract clean-up reported acceptable results for the majority of mycotoxins. Good results were also obtained by laboratories that analysed crude extracts although a high variability of results was observed for all tested mycotoxins. Matrix-matched calibration or isotope-labelled internal standards efficiently compensated matrix effects efficiently whereas external calibration only gave reliable results in cases where less than 10 mg of matrix equivalent amounts were injected onto the HPLC column.

Unacceptable high recovery and high variability of fumonisins results were obtained by the majority of laboratories that could not be explained thus requiring further investigations. These findings provided a great deal of information on currently used methodologies enabling a deep understanding on the effectiveness of different LC-MS-based approaches for mycotoxins analysis in maize.
DETECTION – L5

A promising grain sampling technique: the sampling of wheat dust and subsequent UPLC-MS/MS analysis

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For the analysis of mycotoxins in grains, sampling is the most critical step of the whole analytical process. The sample selection can be made easier and the error of uncertainty lower by the sampling of dust instead of cereals. It is known that there is an accumulation of mycotoxins on small particles for example dust. Dust is created when grain is transported through closed systems and it also accumulates in mills and storage facilities. Because of the correlation between the contamination of the overall sample and the dust particles, this technique results in a fast and reliable decision on the acceptance or rejection of grain lots.

A UPLC-MS/MS method was developed and validated for the determination of deoxynivalenol (DON) in wheat dust. An extraction was performed with acetonitrile/water/acetic acid (79/20/1, v/v/v) followed by a hexane defatting step. After filtration, the extract was evaporated to dryness and the residue was dissolved in mobile phase for injection. The use of a clean-up step was investigated, however because of labor intensive and expensive characteristics, this extra step in the sample preparation was not performed. The analysis was carried out within 8.5 min per run using a Waters Acquity UPLC system coupled to a Quattro Premier XE mass spectrometer (Waters, Milford, MA, USA). The method was validated in terms of linearity, apparent recovery, limit of detection (LOD), limit of quantification (LOQ), precision and expanded measurement uncertainty. The LOD and LOQ were respectively 358 ng g⁻¹ and 717 ng g⁻¹. The results of the performance characteristics of the developed UPLC-MS/MS method were in good agreement with the criteria mentioned in Commission Decision 2002/657/EC.

In the future, the UPLC-MS/MS method can be adapted to the analysis of other mycotoxins in wheat and also in other dust matrices.

Acknowledgement
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DETECTION – L6  

A LC-MS/MS method for the simultaneous determination of zearalenone, deoxynivalenol and metabolites in pig serum

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Zearalenone (ZEN) and deoxynivalenol (DON) are of special importance in animal nutrition due to the frequent contamination of cereal grains and their toxic effects especially on pigs. The diagnosis of intoxications of farm animals might include the analysis of toxin residues in physiological specimens. Unfortunately, only few methods for the simultaneous determination of ZEN, DON and their metabolites alpha- and beta-zearalenol (ZEL), zearalanone (ZAN), alpha and beta-zearalanol (ZAL) and de-epoxy-DON in physiological samples are currently available. Therefore, we developed and validated a selective and sensitive LC-ESI-MS/MS method for pig serum combined with an economic sample preparation.

For development of the sample preparation method 14 columns including SPE columns (based on silica or polymer sorbent), immunoaffinity columns and Chem Elut based on diatomaceous earth were tested for their suitability according to manufacturer’s prescription. The most suitable were selected and the applicability of labeled internal standards (IS) was tested as well as different washing and elution solvents. Finally, sample preparation was carried out using Oasis HLB SPE columns (Waters) as follows: to 500 µl serum 20 µl IS working solution (13C18-ZEN, alpha-ZEL-d4, beta-ZEL-d4, alpha, beta-ZAL-d4 and 13C15-DON) was added and incubated with β-glucuronidase overnight. The sample was loaded onto a preconditioned Oasis HLB SPE column, washed with 2 ml 5% MeOH and eluted with 2 ml MeOH. The eluate was evaporated to dryness and resuspended in 500 µl MeOH/water (70/30 v/v).

Measurements were performed on a 4000 QTrap LC/MS/MS system (Applied Biosystems) coupled with a 1200 series HPLC system (Agilent Technologies). Analytes were separated on a Pursuit™ XRs Ultra 2.8 C18 column within 20 minutes using a binary gradient with water as eluent A and methanol/acetonitrile (70/30 v/v) as eluent B.

The method was in-house validated for all analytes based on the guidelines of the FDA and European community. Calibration graphs (0.3-480 ng/ml) were prepared and good linearity was achieved (r ≥ 0.99). The recoveries were in the range of 76-126% with IS and 31-66% without IS. Limits of detection and limits of quantification were between 0.03-0.71 ng/ml and 0.08-2.37, respectively. The results for accuracy and precision fell within the ranges specified.

This method can be used as a multi-biomarker method to assess animal exposure to these mycotoxins and for diagnosis of intoxications. The method has been successfully used for the determination of ZEN, DON and their metabolites in pig serum from feeding trials with practically relevant ZEN and DON concentrations. The applicability for liquor and plasma was also confirmed. Additionally, preparation of more complex matrices such as bile, urine etc. is in progress.

References
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DETECTION – L7

Fast and sensitive LC-MS/MS method measuring human mycotoxin exposure using biomarkers in urine

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There is a growing interest in measuring exposure to mycotoxins by quantifying biomarkers in urine. Mycotoxin glucuronides are important metabolites and were often measured using indirect methods. After hydrolysis of the glucuronides, either enzymatically or chemically, the parent mycotoxins were quantified. These methods are very laborious, all structural chemical information (isomers) is lost and without standards validation of these methods is difficult. Recently several methods were published using direct quantification of biomarkers in urine. As very low limits of quantification are required and standards are not commercially available, method developing is challenging. Njumbe et al. (2012) successfully developed a method with extensive cleanup and hence risking analyte loss. Warth et al (2012) used the dilute and shoot approach and tentatively identified deoxynivalenol-15-glucuronide (DON-15GlcA) as the major DON-metabolite in human urine. We developed a direct LC-MS/MS method using electrospray negative ionization for glucuronides of: DON, acetyl-DON’s, deepoxyrivalenol (DOM-1), zearalenone (ZEN), alpha- and beta-zearalenol (alpha- and beta-ZEL). Using the same sample extract, we measured the parent toxins in electrospray positive ionization. The sensitivity of this method was compared to indirect methods using either beta-glucuronidase enzymes or chemical hydrolysis. We clearly identified DON-15GlcA as the major DON-glucuronide in human urine, while DON-3GlcA was present in minor amounts. The possible presence of a third DON-glucuronide was explored. In total our method can detect 33 possible biomarkers for mycotoxin exposure. The method was used to measure biomarkers for mycotoxin exposure in urine samples of a small number of volunteers.

References
DETECTION – L8
Fluorescence polarization immunoassays for the determination of mycotoxins in foodstuffs

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Fluorescence polarization (FP) immunoassay is a homogenous assay that measures competition between a fluorescently labelled antigen (tracer) and unlabelled antigen in solution for binding a specific antibody. The FP signal is inversely related to the antigen content that competes with the tracer, and it increases when the binding of specific antibody to the tracer increases. The selection of the appropriate antibody-tracer combination determines the speed, accuracy, precision and sensitivity of a FP immunoassay. Incubation times, cross-reactivity, compatibility with organic solvents and matrix effects are the analytical parameters to be evaluated and optimized in the development of a FP immunoassay. The technology was first developed in the 1970s and has been extensively used in human and veterinary diagnostics. Its application to mycotoxin analysis is more recent and is mainly addressed for screening purposes due to its simplicity, rapidity, cheapness and reliability. FP immunoassays have been recently reported for rapid determination of the major mycotoxins, i.e. aflatoxins, fumonisins, deoxynivalenol (DON), ochratoxin A (OTA), zearalenone, T-2 and HT-2 toxins, in different matrices (unprocessed cereals, cereal-based products, peanut-based products and wine). However these immunoassays in some cases showed low sensitivity and gave results in disagreement with those obtained with more robust HPLC reference methods, due to the presence of matrix interferences. An accurate validation for these assays should be performed on each tested matrix using either artificially and naturally contaminated samples, and reference materials, if available.

An overview of FP immunoassays for mycotoxin detection will be presented focusing on assays that have been developed recently at CNR Institute of Sciences of Food Production (CNR-ISPA) for the determination of DON in wheat and derived products, OTA in red wine and wheat, T-2 and HT-2 toxins in wheat, barley, oats and oatflakes. Advantages, limitations and future perspectives of each method will be discussed.

Acknowledgement
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Quantum dots (QDs) are inorganic fluorescent semiconductor nanocrystals, exhibiting size-dependent fluorescence emission spectra. Due to their unique optical properties (such as high photostability, broad excitation spectra et al.) QDs find use as biolabels in bioanalysis applications. Recently it was shown that QDs-based immunoassay has sensitivity comparable with enzyme-based immunoassay, and it’s necessary to note that the use of QDs as labels significantly decreases time of analysis. Furthermore QDs of different colours can be effectively excited by one wavelength what dramatically simplifies multiaassay realization.

As a rule highly fluorescent QDs for use as biolabels are prepared at high temperature in organic solutions. So, the surface of such QDs are covered with a hydrophobic ligand shell (fig.). Until now the most difficult stage in QDs preparation is still a phase transfer of QDs from organic to aqueous solution because the fluorescence brightness of QDs often dramatically decreases as a result. In this work we synthesized hydrophobic core-shell QDs CdSe/ZnS and CdSe/CdS/ZnS with fluorescence from 520 to 650 nm (fluorescence quantum yield more than 30%) by organometallic synthesis in octadecene. The obtained QDs covered with octadecylamine were transferred to aqueous solution with amphiphilic polymer (fig.): hydrophobic chains of the polymer interact with the surface ligands of the QDs and hydrophilic exterior ends provide water-solubility. In this case the initial hydrophobic ligands aren’t removed from QDs, so the QDs surface is not damaged in this procedure and fluorescence quantum yield only slightly decreases. Amphiphilic polymers were synthesized from inexpensive and stable reagents: poly(maleic anhydride-octadecene) (PMAO, Aldrich, M 30000-50000 g/mol) and polyetheramine (Huntsman). Maleic anhydride groups of PMAO easily react with amine groups of polyetheramine. Depending on polyetheramine composition (fig.) we could obtain QDs with carboxylic or amine groups available for conjugation with biomolecules. It was shown that hydrophilic QDs covered with the synthesized polymers keep up to 95% of the initial fluorescence brightness. The obtained QDs were successfully tested as fluorescent biolabels in microplate immunoassay for mycotoxin zearalenone detection.

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**DETECTION – L9**

**Quantum dots for use as labels for mycotoxin detection: preparation of hydrophilic quantum dots by encapsulation with polymer**

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QDs in organic solvent: covered with octadecylamine QDs in aqueous solution: encapsulated with amphiphilic polymer

Fig. Scheme of a QDs’ phase transfer procedure. R=CH₃ (Jeffamine M1000) and R=NH₂ (Jeffamine ED 2003).
Hapten microarray-based screening of mycotoxins in cereals

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Foodstuffs such as cereals, coffee, spice and wine are often attacked by fungi that can generate toxic substances. Mycotoxins are secondary metabolites of fungi and several of them are known to be acute toxic, immunosuppressive, mutagenic, teratogenic and carcinogenic. The most important mycotoxins in cereals and maize are deoxynivalenol (DON), ochratoxin A (OTA), aflatoxins (esp. AFB1), zearalenone (ZEA), fumonisins (esp. FB1) and T-2/HT-2. Maximum levels for several mycotoxins in food were set by the Commission Regulation (EC No 1881/2006 and EC No 1126/2007). Therefore, the development of fast and sensitive quantification methods for raw food analysis is necessary.

In our institute the automated readout system for flow-through chemiluminescence microarrays, the Munich Chip Reader 3 (MCR 3), was developed during the last decade. This method is based on the indirect competitive ELISA format and allows a multianalyte testing with little or no pre-treatment. The aim of this project is to develop a biochip for the rapid determination of multiple mycotoxins in cereals. In detail, coupleable mycotoxin derivatives are synthesized and then immobilized on PEGylated glass chips yielding a hapten-microarray. The immunological determination takes place in a flow chamber and the chemiluminescence readout is performed by a CCD camera placed on the top.

In the first experiments the regenerability of the chip, immobilized with OTA, AFB1, DON, FB1 and T-2 toxin, was tested. Therefore, 50 cycles with blank wheat extract were run. The results showed that the signal intensities remained higher than 84%. After performing the first calibrations of each mycotoxin in spiked oat extracts, an interday study was carried out to test the intermediate precision of the new method.

The results showed that the standard deviations of the IC\textsubscript{50} values of all mycotoxins were less than 10 %. Furthermore, the generated calibrations allowed a quantification within the regulation limits, especially for AFB1 (WR: 2.4 - 11.4 µg/kg, LOD: 0.9 µg/kg) and OTA (WR: 1.8 - 15.5 µg/kg, LOD: 1.1 µg/kg).

To demonstrate the feasibility of the system, recoveries of different self-spiked samples (oat, wheat, maize) as well as native contaminated samples were determined. The calculated recovery rates (RR) were 82 - 120% for OTA, 75 - 81% for AFB1, 69 - 108 % for T-2 toxin, 55 - 80% for FB1, and 58 - 79% for DON, respectively. To obtain higher RR especially for DON and FB1 different extraction methods were tested with traditional ELISA and compared with the biochip.

**Figure 1:** Interday study for aflatoxin B1 in oat extract carried out on 3 different days.

References:

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Matrix binding of Ochratoxin A during thermal processing

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Ochratoxin A (OTA) is a mycotoxin occurring in a broad range of commodities such as coffee, cocoa, cereal, grapes and spices. During food processing, a reduction of OTA levels can be observed. Especially during coffee roasting, a degradation rate up to 90% is described [1].

Regarding the reduction of OTA levels during roasting there are two chemical degradation reactions known so far, the isomerization to 14-(R)-ochratoxin A and the decarboxylation to 14-decarboxy-ochratoxin A [2]. Besides these degradation reactions the binding of OTA to matrix components under thermal treatment might also be possible resulting in reduced OTA levels in roasted coffee. Such matrix binding of mycotoxins has already been described for fumonisins where the side chains react with matrix components during thermal processing [3].

The aim of the present study was to get more information about the possible binding of OTA to matrix compounds such as polysaccharides like cellulose and hemicellulose during roasting. Therefore model roasting experiments with different matrices were carried out. To determine any binding between matrix compounds and OTA an enzymatic cleavage of the polysaccharide backbone was performed in order to obtain low molecular weight OTA binding products, which were then analyzed by high performance liquid chromatography coupled with fluorescence detection and Fourier transform mass spectrometry (HPLC-FLD-FTMS) and with tandem mass spectrometry (HPLC-MS/MS).

References
PRODUCTION AND OCCURRENCE – L12

Masked mycotoxins: facts and figures 2009-2013

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Metabolisation of mycotoxins by plants can partially occur, and gives rise to the production of so called “masked mycotoxins”. The possible hydrolysis of masked mycotoxins into their toxic parent forms during mammalian digestion is a potential risk for consumers.

Between 2009-2013 masked mycotoxins have received increased attention in view of their bioavailability, occurrence and potential toxicity in animals and humans, however their presence and relevance in the field remain undisclosed. In this presentation an overview is presented of recent developments on the masked mycotoxin problemacy accompanied with own results of the past 4 years.

Foodstuffs which are susceptible to Fusarium contamination include wheat, maize, barley and cereal-based products such as breakfast cereals, bread and beer. Furthermore, in these matrices co-occurrence of masked and parent mycotoxins has been described. Also, a positive correlation between these parent and masked forms was established. This indicates that a higher contamination of a particular mycotoxin is coupled with an elevated load of the other.

To date however, no risk assessments were performed for masked mycotoxins. A quantitative dietary exposure assessment of mycotoxins and their masked forms was conducted on a national representative sample of the Belgian population using the contamination data of cereal-based foods (n = 174). The occurrence of deoxynivalenol, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, zearalenone, α-zearalenol, β-zearalenol, T-2-toxin, HT-2-toxin, and their respective masked forms, including, deoxynivalenol-3-glucoside, zearalenone-4-glucoside, α-zearalenol-4-glucoside, β-zearalenol-4-glucoside and zearalenone-4-sulfate was investigated via liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS).

Although the majority of the Belgian population did not exceed the total daily intake of 1.0, 0.2 and 0.1 µg kg−1 bw day−1 deoxynivalenol, zearalenone and the sum of T-2-toxin and HT-2-toxin, respectively, there was a large subpopulation, exceeding those safety values.

The use of high resolution mass spectrometry (HRMS) made it possible to identify new masked Fusarium forms such as the glucosides of T-2-toxin, HT-2-toxin, fusarenon-X, nivalenol and neosolaniol. The importance of these newly discovered forms is demonstrated by showing occurrence data of cereal-based foods from 2013.

All the mentioned results highlight the importance to screen for multiple mycotoxins, both parent and masked in order to guarantee food and feed safety.

Acknowledgments
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PRODUCTION AND OCCURRENCE – L13

Correlation of ergot alkaloids and sclerotia in German rye and wheat and recommendation to simplify analyses

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Fungi of the genus Claviceps produce sclerotia instead of a grain kernel which may contain a wide range of bioactive compounds, e.g. ergot alkaloids. The scientific opinion published by the EFSA in 2012 advised to determine the contents of the most important ergot alkaloids amongst others in cereals [1]. The aim of the investigation was first to examine the levels and types of ergot alkaloids in German rye and wheat samples of harvests 2010 and 2011 and second to correlate them to the overall ergot alkaloid and sclerotic content, respectively.

The rye and wheat samples were collected during the German official harvest survey called “Besondere Ernte- und Qualitätsermittlung BEE”. About 500 rye and nearly 900 wheat samples of the harvests 2010 and 2011 were analyzed. After grinding (500μm) the samples were screened for ergot alkaloids with an LC-MS/MS-method [2]. Positively screened samples were further analyzed according to the validated method from Müller et al. [3].

Results: There were fewer samples tested positively for ergot alkaloid in 2011, but the total ergot alkaloid contents were higher compared to 2010. Comparison of the two years of harvesting shows various significant differences between the German states in the statistical evaluation. Analyses do not show any significant differences between the diverse rye and wheat varieties neither in the harvest year 2010 nor in 2011.

We did not find any correlation between the total ergot alkaloid content and the sclerotic content in rye samples. In contrast various correlations arose between the total ergot alkaloid content and the contents of different ergot alkaloids, but it was not possible to specify lead substances. Good correlations also occurred between the values of ergotamine, ergocornine, ergocryptine and ergocristine and their 8-(S)-diastereomers in rye and wheat samples in both years of harvest letting us conclude, that it might be possible to calculate the -nime form from the corresponding -ine content. Results of the calculation method will be discussed as well as advantages and limitations.

References
PRODUCTION AND OCCURRENCE – L14

Screening biodiversity of toxigenic fungi for a correct evaluation of mycotoxin risk on important food crops: the contribution of EU project MycoRed, at a global level.

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To study the biodiversity of toxigenic fungi occurring on food crops provide information on the correct evaluation of the mycotoxicological risks related to their occurrence. The importance of surveys on toxigenic fungi biodiversity is also related to the changes of environmental conditions worldwide, which significantly influence the distribution and the profile of specific toxigenic fungi. In addition, trans-global transposition and trade exchanges of plant products contribute to the spreading of toxigenic fungi worldwide. MycoRed is contributing to the biodiversity studies by the collection of a wide number of fungal strains belonging to \textit{Aspergillus}, \textit{Fusarium}, and \textit{Penicillium} genera and isolated from samples of cereals (mainly maize and wheat), grape and dried fruits from different countries worldwide.

The biodiversity studies of strains are based on both a multi-locus phylogenetic approach by sequencing part of the $\beta$-tubulin ($\beta_t$), calmodulin (caM), RNA polymerase II (RPB2) and elongation factor 1$\alpha$ (EF-1$\alpha$) genes and the analysis of their toxin production. A relevant increase of the fungal collection of Institute of Sciences of Food Production (ITEM collection) has been obtained during the project: over 5000 strains, 3000 strains of which isolated from the food commodity samples at global level. The identification of strains from natural samples has led to a total of 1400 strains belonging to \textit{Aspergillus}, 400 strains to \textit{Penicillium}, and 1200 strains belonging to \textit{Fusarium} genus. \textit{Aspergillus} section Nigri isolates were predominant with respect to \textit{Aspergillus} section Flavi strains. The strains of the \textit{Penicillium} genus belonged mostly to the Subgenus \textit{Penicillium} (therverticillata) in sections: \textit{Penicillium}, \textit{Viridicata}, \textit{Coronata} and \textit{Roqueforti}. On the other hand, among strains belonging to \textit{Fusarium} genus, the most relevant number was collected from cereals (maize, oat, wheat), but also dried figs and other sources. The strains isolated from oat and wheat belonged mostly to species that produce the highly toxic trichothecenes of both type A (T-2 and HT-2 toxins) and type B (deoxynivalenol and nivalenol), and some minor mycotoxins such as beauvericin, enniatins, and moniliformin. In particular, strains belonging to \textit{F. graminearum} species complex and to species of the former \textit{Gibbosum}, \textit{Roseum} and \textit{Sporotrichiella} sections, were identified. The strains isolated from maize and dried figs mainly belonged to the \textit{Fusarium fujikuroi} species complex, that include fumonisins producing species. Moreover, representative strains for each species or population, phylogenetically determined, were analyzed for their mycotoxin production in order to define a typical mycotoxin profile for each group of fungal strains. The three years of MycoRed survey led us to detect a great biodiversity of the toxigenic fungal strains at global level for both the range of species collected from the different crops and the potential of their multi-toxin production. Such biodiversity shows that the risk related to mycotoxins in food commodities is due not only to a single specie, but more often is related to the occurrence of groups of toxigenic fungi that can be genetically closely related or distant. Therefore, this complexity requires deeper investigations on the potential additive and/or synergistic effects of the combinations of more mycotoxins on a single crop. Finally, the great diversity of the fungal strains deposited in ITEM collection will guarantee a unique biological source for deep worldwide investigation on both new emerging toxigenic fungal species and new mycotoxin/commodity combinations.

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Fungal growth and mycotoxin production during cocultivation of phytopathogenic *Fusarium* and *Alternaria* strains

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Fungi of the genera *Fusarium* and *Alternaria* attack and infect their cereal host plants in the field, mostly in the period from the anthesis to the mid-grain-filling stage. It is well documented that the infection and colonisation of plants by these pathogens are strongly favoured by high humidity and that the species-specific mycotoxins can play an important role in their plant-pathogenesis. Therefore, a co-occurrence of *Fusarium* and *Alternaria* in wheat ears is very likely as is the co-occurrence of different mycotoxins. This implies a potential risk of additional or even synergistic toxic effects on human and animals after consumption of contaminated cereal or cereal products.

A laboratory study was conducted to evaluate the influence of co-cultivation of toxigenic *Fusarium* (*F*) and *Alternaria* (*A*) fungi with respect to growth and mycotoxin production. *F. culmorum* Fc13, *F. graminearum* Fg23 and two *A. tenuissima* isolates (At18 and At220) were simultaneously or consecutively co-incubated on wheat kernels in an *in vitro* test system. Fungal biomass was quantified by determining ergosterol content. Three *Fusarium* toxins (DON, NIV and ZON) and three *Alternaria* toxins (AOH, AME and ALT) were analyzed by a newly developed HPLC/MS/MS method.

Co-cultivation significantly affects fungal growth and mycotoxin production of phytopathogenic *Alternaria* and *Fusarium* strains. In simultaneous co-cultures the fungal biomass was enhanced up to 460% compared to individual cultures; *Alternaria* toxins were considerably depressed down to <5%. Combining At18 and At220 with Fg23 inhibited the toxin production of both fungal partners. In contrast, Fc13 increased its DON and ZON production in competitive interaction with both *A*. strains. In the consecutive inoculation, the second fungal partner in cocultures was barely able to produce the species-specific mycotoxins. The firstly inoculated *Alternaria* strains remarkably increased their species-specific mycotoxin production in the competition with the consecutively inoculated fusaria.

The inter-fungal competitive effects aid the understanding of the processes of competition of both fungi in natural environments and the involvement of mycotoxins as antifungal factors.

**References**

Interaction of free air carbon dioxide enrichment (FACE) and water availability on deoxynivalenol concentration in maize and sorghum

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The atmospheric CO₂ concentration [CO₂] is predicted to rise from actual 385 µl l⁻¹ up to 550 µl l⁻¹ until the middle of this century. As a consequence an increase of air temperature and more frequent summer droughts are expected for large parts of Central Europe. These changes will have an important impact on cropping systems because growth and health of cultivated plants are decisively influenced by climate and weather conditions.

Maize and sorghum are important C₄ crops not only in terms of global food and feed, but also biofuel production. There is evidence that an elevation of atmospheric CO₂ decreases the transpiration demand of C₄ crops and thus may mitigate the negative effects of limited water availability under drought stress conditions. Major fungal pathogens causing infectious diseases endangering food and feed safety by mycotoxin production in both maize and sorghum belong to the genus *Fusarium*. In cooler regions of Europe the species *F. graminearum* and *F. culmorum* producing deoxynivalenol (DON) are the most important toxigenic species infecting cereals and maize in the field.

As a special part of the first FACE experiment carried out in Europe with C₄ crops (Manderscheid et al., 2012) this study aimed to evaluate the potential interactive effects of CO₂ enrichment and summer drought on DON contamination of maize and sorghum. The trial was conducted on an experimental field site of the Thünen Institute (Braunschweig). The FACE system consisted of three rings each with a diameter of 20 m to reach a target [CO₂] of 550 µl l⁻¹ during daylight hours beginning from June until the end of season. Three circular plots without CO₂ enrichment served as control (ambient [CO₂]). Each area of both FACE and control rings was split into a semicircle with sufficient (WET) and restricted water supply (DRY). The DRY treatment was achieved by rainout shelters which were used to exclude rainfalls. Maize was grown during four seasons in the years of 2007/2008 (cultivar ‘Romario’) and 2010/2011 (cultivar ‘Simao’), whereas sorghum (*Sorghum bicolor* × *bicolor* cvs. ‘Bulldozer’ and ‘Zerberus’, *Sorghum bicolor* × *sudanese* cv:’Inka’) was grown during the seasons of 2010 and 2011. Whole plants of both crops were harvested by hand at maturity (growth stage: BBCH 83-85), subsequently chopped, oven dried and ground (particle size: ≤ 1 mm) before being analysed for DON by use of ELISA test kits from R-Biopharm, Darmstadt, Germany.

Due to huge rainfall in 2010 and especially in 2007, which could not be excluded from the DRY subplots to limit water availability for the plants, heavy drought stress could only be established in 2008 and 2011 when DRY plots received approximately half the amount of water as compared to the WET plots. DON concentrations differed largely between species, cultivars and years and ranged from 100-600 µg kg⁻¹ in the sorghum cultivars up to 9000 µg kg⁻¹ in the maize cultivar ‘Romario’. With respect to DON concentrations detected in maize regarding all seasons, no clear differences were observed between both enriched [CO₂] compared with ambient [CO₂] and WET compared with DRY treatments. Similar lack of effects derived from different treatments of sorghum grown in 2010, while in 2011 DON contamination of sorghum was increased at enriched [CO₂] compared with ambient [CO₂] both at well-watered and drought stress conditions.

Reference
PRODUCTION AND OCCURRENCE – L17

Upregulation of sterigmatocystin and other putative mycotoxins in Aspergillus nidulans after feeding damage by Folsomia candida

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The soil ascomycete Aspergillus nidulans produces numerous secondary metabolites. Sterigmatocystin, a mutagenic and carcinogenic mycotoxin similar to aflatoxins, is the most investigated secondary metabolite produced by the fungus. Secondary metabolites were suggested to act as resistance agents against invertebrate fungivores (Rohlfs and Churchill, 2011). Collembola are highly abundant microarthropods feeding on fungal hyphae which can drastically reduce the fungal biomass (Bretherton et al., 2006). Mycelia of A. nidulans colonies treated (Fig. 1) and not treated with F. candida were analyzed for the content of secondary metabolites using non-targeted metabolic profiling by HPLC-MS. Mass spectra and retention times were used for the identification of metabolites enhanced by feeding. Sterigmatocystin was quantified using external standards. We found a significant up-regulation of sterigmatocystin and further secondary metabolites identified as austinol, dehydroaustinol and emericellamides C, D, E and F. Our study suggests that these secondary metabolites are involved in chemical defense response of A. nidulans to fungivores. The role of these metabolites in the interaction and their effects on fungivores remain to be determined.

Fig. 1: Collembola Folsomia candida feeding on mycelia of Aspergillus nidulans

References
PRODUCTION AND OCCURRENCE – L18

Chlorogenic acid, a metabolite identified in tomato fruits by a metabolomics approach, is inhibitory against the biosynthesis of alternariol by Alternaria alternate

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Tomato fruits and processed tomato products are often reported to contain the mycotoxins alternariol (AOH), alternariol monomethyether (AME) or tenuazonic acid (TEN). All three mycotoxins are produced by the fungus A. alternata. Tomato products generally contain moderate to low concentrations (Ackermann et al., 2012) but may occasionally contain very high amounts of these toxins (Ostry, 2008). According to the former publication the occurrence of AOH in tomatoes seems to be a common problem. These results show that tomatoes are a typical habitat for A. alternata and that the production of AOH, AME or TEN is highly dependent on the environmental conditions, e.g. the substrate composition of tomato varieties. Based on these facts tomato as substrate might either be more supportive or reductive for the biosynthesis of AOH depending on the variety. In fact in a laboratory experiment a strain of A. alternata showed a reduced production of AOH on a tomato model medium compared to other media. This result indicates that despite the fact that tomato is a common substrate for A. alternata, it might contain AOH inhibiting substances. For this reason an untargeted metabolite profiling of various tomato varieties was performed by GC x GC/MS analysis. Tomato fruits of the same set of varieties were infected with A. alternata in parallel. One variety proved to be more resistant against growth and AOH biosynthesis of A. alternata. Metabolome data visualization using volcano plots revealed that among others, the concentration of chlorogenic acid was substantially higher in this variety. In subsequent growth experiments it could be demonstrated that purified chlorogenic acid indeed has a concentration dependent moderate growth inhibiting, but a strong AOH biosynthesis inhibiting effect. Transcriptional analysis of AOH biosynthesis genes by Real Time PCR revealed a correlation between gene expression and AOH biosynthesis. These results indicate that chlorogenic acid might be one of the metabolites, which reduce alternariol biosynthesis in more resistant tomato varieties. Beside the reduction of AOH biosynthesis also a reduced colonization could be observed on this tomato genotype. This is in agreement with the recent observation that AOH is a pathogenicity or colonization factor. The presence of increasing AOH concentrations led to stronger colonization which seems to be counteracted by the presence of increased amounts of chlorogenic acid.

References


**New secondary metabolites of *F. fujikuroi* – a combined biological and chemical approach**

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*Fusarium fujikuroi* is a common pathogen of rice and well-known for its ability to generate gibberellic acids that are responsible for the so called “Bakanae” disease of rice [1, 2]. Besides these phytohormones, *F. fujikuroi* is able to produce further secondary metabolites such as beauvericin, fusarins, fusaric acid, fumonisins B₃, moniliformin, bikaverins and fusarubins and has therefore also to be considered concerning food safety aspects [2].

Recently, the genome of *F. fujikuroi* IMI58289 has been sequenced and analyses of the genome revealed that the fungus possesses the ability to produce a larger number of secondary metabolites than the ones known so far [3]. In detail, there are 17 polyketide synthase (PKS) and 15 non-ribosomal peptide synthase (NRPS) gene clusters that can be assigned to only 6 of the known secondary metabolites [3]. Of the remaining clusters, especially one PKS and one NRPS gene cluster are of high interest, as they are unique for *F. fujikuroi*. Thus, looking at the products of these unique gene clusters is promising to elucidate the structure of so far unknown secondary metabolites [3].

A combined molecular biological and chemical approach was chosen to generate mutants overexpressing specific cluster genes and to monitor the product formation in these mutants by HPLC-FTMS analysis. In detail, the investigation of the unique NRPS cluster is presented describing the challenge and progress starting from the cluster identification and leading to a newly identified secondary metabolite.

**References**


**PRODUCTION AND OCCURRENCE – L20**

**Tenuazonic acid in infant products – a risk for the consumer?**

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**Introduction:** The mycotoxin tenuazonic acid (TA) is produced by fungi mainly belonging to the genus *Alternaria* and has attracted increasing attention since high contents of TA have been found in food commodities such as tomato products, cereals, juices and spices[1]. Regarding its toxicity, TA has been found to inhibit protein biosynthesis, to exert antiviral, antitumor, antibacterial, cytotoxic and phytotoxic properties and also to be acutely toxic in mammals. However, the assessment of adverse health effects is essentially hampered, as no toxicokinetic data about absorption, metabolism and excretion of this mycotoxin are available. In view of its almost ubiquitous occurrence, the goal of this study was to examine the content of TA in infant products and to use the data for assessing the risk imposed by this mycotoxin.

**Results and Discussion:** Different infant foods and beverages including tea infusions, pureed infant food in jars and infant cereals were analysed for TA using a stable isotope dilution assay (SIDA) developed recently [2]. The median content of TA in infant tea infusions (n = 12) was 2 µg/L, but values up to 20 µg/L were found in fennel tea infusions. In puree infant food in jars (n = 12), the median content of TA was 7 µg/kg, but higher values were detected in products containing tomato (25 µg/kg), banana and cherry (80 µg/kg) and sorghum (20 µg/kg). Infant cereals on the basis of wheat and/oats, rice, spelt and barley (n = 4) did not contain TA in values higher than 30 µg/kg, but if sorghum was the major ingredient (n = 12) the mean content of TA was 550 µg/kg and the maximum level was 1,200 µg/kg. The European Food Safety Authority (EFSA) evaluated the toxicological potential of TA by following the threshold of toxicological concern (TTC) approach yielding a TTC value of 1,500 ng TA/kg body weight per day [3]. Although long-term studies are needed to enlarge the database on TA contamination of sorghum based infant food, our preliminary study points out to a tendency that the TTC value may be exceeded by infants consuming predominantly sorghum based food. Therefore, further investigations regarding the toxicokinetic properties of TA in humans are necessary. Studies relating the intake of cereals and urinary excretion are under way.

**References**

The publication of mycotoxin results exceeding the maximum levels in foodstuffs – an increase in market transparency?

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Effective from 1 September, 2012 the German consumer information act (VIG) has a new facet, with far-reaching consequences for the official food control. Article 2 of the updated version stipulates that all data regarding exceedance of maximum levels (ML) in foodstuffs or consumer products must be made accessible to the public. This includes, of course, foodstuffs from the German market which exceed established MLs for mycotoxins or other contaminants and residues. The main aim of the law is to establish market transparency for the consumers. The state of Baden-Württemberg publishes the offenses against EU regulations and the German food law on a web page: www.verbraucherinfo-bw.de (consumer info-Badenwürttemberg).

This presentation will report on the process of publishing data on exceeding mycotoxin residues detected in an official control laboratory since the law has been in effect. It will highlight special publishing cases in the field of mycotoxins and discuss the aim of market transparency as well as difficulties concerning mycotoxins in foodstuffs in connection with the consumer information act.

References
Verbraucherinformationsgesetz (VIG) in der Fassung der Bekanntmachung vom 17. Oktober 2012 (BGBl. I S. 2166, 2725)
PRODUCTION AND OCCURRENCE – L22

Chemical interactions between *Fusarium verticillioides* and *Gliocladium roseum* involving mycotoxin transformation

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The effect of fungal interactions on secondary metabolite production is poorly understood. *Fusarium verticillioides* and *Gliocladium roseum* were grown in dual cultures, DNA of *F. verticillioides* was determined (Fig. 1) and secreted metabolites were analyzed.

Dual cultures accumulated metabolites not produced in single cultures. Purification of selected new metabolites revealed that they were derivatives of fusaric acid (Fig. 2). While compounds C and D have been described before, compounds A and B are new.

![Figure 1. DNA of *F. verticillioides* in single and dual cultures.](image)

![Figure 2. Derivatives of fusaric acid from dual cultures of *F. verticillioides* and *G. roseum*.](image)
**PRODUCTION AND OCCURRENCE – L23**

*Fusarium poae*: chemotype, plant-pathogen interaction and response to oxidative stress triggers

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Fusarium Head Blight is a devastating small-cereal disease, affecting yield and crop quality of such agronomically important crops as wheat and barley. The disease is caused by a blend of Fusarium species, in which *Fusarium poae* has in recent years become more and more important. Species determination in Belgium from 2002 to 2012 showed a steady increase of the *F. poae* presence in the field population.

Sampling in the field and subsequent single spore isolation have led to the creation of an in-house *F. poae* collection. Over the course of a PhD thesis, this collection will be analyzed from different angles to achieve a comprehensive understanding of this pathogen. The use of media that induce trichothecene synthesis in combination with UPLC-MS/MS have led to the identification of up to 5 different chemotypes in the population. The implications of this are discussed in relation to incidence data of several relevant *F. poae* toxins in the field, and in food and feed products, collected over an extensive survey in 2012.

The role of *F. poae* toxins in plant colonization is unknown. Several strategies are presented to elucidate this role in an *in vitro* and *in vivo* approach, including detached leaf and germination assays, and infection studies with pure toxins, and *F. poae* isolates of different chemotypes. Cytological staining presents the means to interpret defense responses of the wheat plant. Where possible, preliminary results are presented.

The much more researched species *F. graminearum* mainly produces the mycotoxin deoxynivalenol (DON). In recent years, a role in fungal defense and tolerance to oxidative stress has been attributed to this compound. Using the application of triazoles, strobilurines and their combination as a model system for oxidative stress, it is possible to research whether *F. poae* behaves in a similar way. Simultaneously, resistance of the pathogen to these fungicides is assessed, and tested as a possible explanation for the increased importance of *F. poae*. Preliminary findings are presented.
Population structure and mycotoxin production of *Fusarium* species from greenhouse pepper fruit in China

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Diseased pepper fruit samples were collected from different provinces in China in 2012. Symptomatic tissues were surface sterilized, dried and then placed in 90-mm Petri dishes containing potato dextrose agar (PDA) and incubated for 5 days at 28 °C. The *Fusarium* strains were single-spore purified and characterized at species level by morphological observations and translation elongation factor 1-α (TEF) gene sequencing. Of the 43 isolates in total, 27 were identified as *F. equiseti*, 10 as *F. solani*, 5 as *F. fujikuroi* and 1 belonged to *F. concentricum*. Completion of Koch's postulates established that all the four *Fusarium* species were the causal agent of greenhouse pepper fruit rot. The results indicate that *F. equiseti* was the predominant pathogenic on pepper in China. To our knowledge, this is the first report of *F. concentricum* associated with pepper fruit rot in China.

Production of the mycotoxins trichothecenes (DON, 3-ADON, 15-ADON, NIV, 4-ANIV, DAS, NEO), fumonisin (FB₁), mycophenolic acid (MPA) and beauvericin (BEA) by the predominant pathogenic *F. equiseti* and *F. solani* was evaluated on rice medium. All the three *F. equiseti* isolates examined were found to produce 4-ANIV and DAS, at concentrations ranging from 168 to 10760 µg/kg and 0.4 to 148 µg/kg, respectively. NEO was produced by two *F. equiseti* isolates ranging from 154 to 2300µg/kg, MPA also were detected in two *F. equiseti* cultures (36 and 72 µg/kg, respectively). Low level BEA (1.2 µg/kg) were produced by one *F. equiseti* isolate. Only MPA (120 to 164 µg/kg) was produced by two of three *F. solani* isolates. No DON, 3-ADON, 15-ADON, NIV and FB₁ were detected in all the culture.

**Acknowledgement**

This work was financially supported by the Shanghai Agriculture Commission Project (2011NO. 4-3) and the Chinese-Belgian Joint Project of BELSPO, Belgium (BL/02/C58) and MOST, China (2012DFG31840).
Impact of monacolin K on animal health: preliminary test of rumen stability

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Silage is of major importance in feeding ruminants. However, it is frequently infected with moulds (1). One of the most common moulds in silage is Monascus ruber, a potent producer of Monacolin K. This fungal compound has been found in considerable concentrations (up to 80 mg/kg) in maize- and grass-silages (2). Monacolin K can bind to hydroxymethylglutaryl coenzyme A reductase, the rate-limiting enzyme in mevalonate synthesis (3). This reversible and competitive effect reduces sterol synthesis in mammalian cells (4) and stigmasteryl synthesis in plants but also ergosterol synthesis in fungi, resulting in antifungal activity. As compounds with antibiotic and antifungal activities could influence the growth and metabolism of rumen micro-organisms like Neocallimastix sp., Monacolin K could possibly affect rumen fermentation. Since cholesterol acts as precursor in steroid hormone synthesis and the exogenic uptake of cholesterol in ruminants is very limited, an influence of Monacolin K on the fertility of livestock might also occur (5).

However, a prerequisite of the proposed effects is the lack of significant microbial degradation in the alimentary tract. Hence several in vitro experiments have been carried out to check rumen stability of Monacolin K.

In the first experiment, a static anaerobic system using rumen fluid from fistulated cattle, Monacolin K showed no degradation during sampling time (48 h). Also in a dynamic anaerobic system (RUSITEC, rumen simulation technique), the added substances showed transformation, Monacolin K (lactone form) was metabolised in the acid form (MKa), but only minor reduction could be observed in the summation of both forms over a period of 24 hours. Since the rumen passage rate of comparable substances usually doesn’t exceed 12 hours in cattle, Monacolin K can be constituted as rumen-stable. Rumen-stability should result in detectable amounts of Monacolin K or metabolites in bovine milk and blood in the field. The LC-MS/MS detection methods developed for this purpose enabled a precise, accurate and sensitive determination of Monacolin Kα and Kβ. In milk, the recovery rates were 99.3 % (MKα) and 94.5 % (MKβ), the limits of detection 0.4 ng/mL and 1.6 ng/mL, respectively. The recoveries in blood were 98.3 % and 83.9 %, respectively.

References
Zearalenone-mediated toxicity in fish \textit{in vitro} and \textit{in vivo}

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This study provides initial data on the toxicity of zearalenone (ZEN) on five permanent cell lines derived from different fish species. Accordingly, ZEN at concentrations ranging from 313 ng ml$^{-1}$ to 20000 ng ml$^{-1}$ influenced cell viability in a dose-response manner with different response patterns between the five tested cell lines. Since three different cell viability tests were used, a specific mechanism of action of ZEN in these cell could be derived from the obtained data that suggests that cellular compartments such as lysosomes are affected before plasma membrane damages occur. The involvement of oxidative stress in the different cell lines which were observed especially at concentration above 7500 ng ml$^{-1}$ ZEN resulting in depletion of antioxidants and increased DNA damages was demonstrated. Moreover, the investigation of cytotoxic effects was accompanied by studies on the metabolism of this mycotoxin in the different cell lines.

The effects of ZEN have also been investigated \textit{in vivo} using carp (\textit{Cyprinus carpio}). Therefore, carp were fed concentrations of 392 to 1147 µg ZEN per kg feed for 4 weeks to show possible effects of this mycotoxin at concentrations below the recommended value of 2 mg kg$^{-1}$ feed material (with the exception of maize by-products) established by the European Commission (2006/576/EC). Haematological and growth parameters were determined to evaluate possible effects of ZEN on whole fish. From our studies it can be concluded that the mycotoxin ZEN exerts several detrimental effects on fish and fish cells.

References
Deoxynivalenol (DON) and fumonisins (FB) are important mycotoxins produced by Fusarium species and commonly co-occur in animal diets. Over 54% of cereal samples collected from European countries were contaminated with both DON and FB (Monbaliu et al., 2010). Previously, we showed a predisposing effect of DON on subclinical necrotic enteritis (NE) in broilers. Cladostrium perfringens induced NE is a major problem in the worldwide broiler industry, leading to significant production losses. The predisposing effect could be contributed to DON affecting the intestinal barrier function. Fumonisins on their behalf, inhibit the glycosphingolipid biosynthesis, and as such have a negative effect on the intestinal integrity and intestinal epithelial renewal. The objective of this study was to evaluate the effect of co-occurrence of the mycotoxins DON and FB, at concentrations approaching the European guidance levels, on the predisposing effect on NE.

The study was conducted in triplicate using a subclinical necrotic enteritis model (Gholamiandehkordi et al., 2007). Per replicate 120 one-day-old Ross 308 broilers were randomly divided into four groups. Throughout the entire experiment, group 1 received a mycotoxin blank diet, while groups 2, 3 and 4 received a mycotoxin contaminated diet. The diet of group 2 was experimentally contaminated with approximately 20,000 µg FB1+FB2/kg feed, group 3 was fed a diet contaminated with DON at a concentration of approximately 5,000 µg/kg feed. The last group was fed the combination of both mycotoxins at similar dosages. All birds were challenged orally with C. perfringens NetB positive strain 56 for four consecutive days starting at day 17. At 1, 2 and 3 days after the final challenge with C. perfringens, 10 chickens per group per day were euthanized and scored macroscopically for intestinal NE lesions (Keyburn et al., 2006).

Results will be presented at the conference.

References
Deoxynivalenol (DON) is a *Fusarium* mycotoxin that frequently contaminates broiler feed throughout the world. The avian immune system is sensitive to mycotoxins. In the present study we investigated the effects of DON and a detoxifying agent on the expression of immune genes of broilers. We employed the following dietary groups: 1) control group fed basal diet, 2) DON group fed basal diet contaminated with 10 mg DON/kg diet, 3) DON+ Mycofix group fed basal diet contaminated with 10 mg DON/kg diet and supplemented with 2.5 kg of Mycofix Select (MS) (Biomin GmbH, Herzogenburg, Austria) per ton of diet, and 4) Mycofix group fed basal diet supplemented with 2.5 kg of Mycofix Select (MS) per ton of diet. At the age of 35 d the birds were slaughtered and 5 cm from mid-jejunum was immersed in the RNA stabilization reagent, RNAlater® (Qiagen, Hilden, Germany) for subsequent quantitative RNA assays of tumour necrosis factor alpha (TNFα), interleukin 8 (IL-8), interleukin 1 beta (IL-1β), nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (TGFBR1), interferon gamma (IFNγ), transforming growth factor, beta receptor I (TGFBR1) and nuclear factor kappa-light-chain-enhancer of activated B cells 1 (NF-κB1) by RT-PCR. The results show that feeding DON-contaminated diet down-regulated the gene expression of IL-1β, whereas the addition of Mycofix up-regulated IL-1β to values comparable to controls. The gene expressions of IL-8 and NF-κb were up-regulated in group 3, as well as that of TNFα in group 4. IFN-γ gene expression was down-regulated due to DON feeding and MS addition.

These results suggest that DON altered the gene expression of important immune response genes which are able to impair the immune functions of broilers. Additionally, Mycofix Select supplementation

### Table 1: Relative expression of immune relevant genes of broilers fed deoxynivalenol and counteragent Mycofix Select

<table>
<thead>
<tr>
<th>Gene</th>
<th>Groups (2, 3, 4)</th>
<th>Relative expression</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNFα</td>
<td></td>
<td>1.14</td>
<td>0.17</td>
</tr>
<tr>
<td></td>
<td>DON</td>
<td>1.17</td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td>DON + Mycofix</td>
<td>1.31</td>
<td>0.07</td>
</tr>
<tr>
<td></td>
<td>Mycofix</td>
<td>1.11</td>
<td>0.26</td>
</tr>
<tr>
<td>IL-8</td>
<td></td>
<td>1.49</td>
<td>0.19</td>
</tr>
<tr>
<td></td>
<td>DON</td>
<td>1.19</td>
<td>0.42</td>
</tr>
<tr>
<td></td>
<td>DON + Mycofix</td>
<td>0.54</td>
<td>0.07</td>
</tr>
<tr>
<td></td>
<td>Mycofix</td>
<td>1.21</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>Mycofix</td>
<td>0.90</td>
<td>0.29</td>
</tr>
<tr>
<td>IL-1β</td>
<td></td>
<td>0.83</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>DON</td>
<td>1.12</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
<td>DON + Mycofix</td>
<td>0.82</td>
<td>0.17</td>
</tr>
<tr>
<td>TGFBR1</td>
<td></td>
<td>0.36</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>DON</td>
<td>0.46</td>
<td>0.04</td>
</tr>
<tr>
<td>IFNγ</td>
<td></td>
<td>0.36</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>DON</td>
<td>1.23</td>
<td>0.19</td>
</tr>
<tr>
<td></td>
<td>DON + Mycofix</td>
<td>1.65</td>
<td>0.13</td>
</tr>
<tr>
<td>NFKB 1</td>
<td></td>
<td>1.71</td>
<td>0.16</td>
</tr>
</tbody>
</table>

* In relation to GAPDH; GAPDH was chosen as a reference gene, having the same relative expression mean in the control as in the treated groups.
changed the effects of DON on the expression of some immune genes.

**IMPACT ON ANIMAL AND HUMAN HEALTH – L29**

**Detection of *Fusarium* mycotoxins and carry over to animals and humans: case study poultry**

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Contamination of food and feed products by mycotoxins is a widespread problem and has resulted into specific legislation or recommendations for certain compounds. Over the last years, it has become clear that *Fusarium* species can also produce other mycotoxins like enniatin B (ENN B), B1, A and A1, beauvericin (BEA), fusaproliferin and moniliformin. Because these emerging toxins are often present in concentrations higher than those of the classic mycotoxins, more data are needed to correctly evaluate the potential risk for the consumer. In this study, transfer from feed to eggs and chicken tissues was studied for deoxynivalenol (DON), T-2 toxin (T-2), HT-2 toxin (HT-2), zearalenone (ZEA), enniatins and BEA. Contaminated feed was prepared making use of a reference material (RM) containing high concentrations of the mycotoxins studied. This RM was produced after identification of *Fusarium* mycotoxin superproducers. Control feed contained mycotoxins in lower concentrations, due to unavoidable natural contamination. Eighty broilers received control feed (starter) during 12 days. After this period, 40 animals received the control feed (grower-finisher) till slaughter age. The 40 other broilers received the artificially contaminated feed during 14 days. Afterwards, all animals received the control feed again during 14 days. Sampling of 6 broilers occurred at 5 occasions: age of 20, 27, 29, 34 and 40 days. The different matrices (meat, liver and skin) were homogenized and kept at -20°C until UHPLC-MS/MS analysis (see presentation “Development and validation of a multi-mycotoxin UHPLC-MS/MS method for the detection of *Fusarium* mycotoxins in eggs and poultry meat”).

Laying hens were selected on body weight and zootechnical performance. In total 36 laying hens were used: 18 for the control group and 18 for the treatment group. Contaminated feed was supplied during 14 days, followed by a depletion period (control feed) of 14 days. During the steady state period, eggs were gathered on a daily base, homogenized and kept at -20°C until analysis.

Carry over (percentage) was calculated as the concentration of mycotoxin found in the animal matrix divided by the concentration of mycotoxin present in the feed given.

In thigh muscle, breast muscle, liver and skin, no 3-acetyl-DON, DON, de-epoxy-DON (DOM), α-/β-zearalenol (α-/β-ZOL), T-2 or HT-2 could be detected. Quantifiable results were only obtained for ENN B, ENN B1 and BEA with highest concentrations for BEA. Concentrations in skin were the highest followed by liver, thigh muscle and breast muscle. As an example, carry over values for thigh muscle were 0.04 %, 0.04 % en 0.26 % for ENN B, ENN B1 en BEA, respectively.

In the eggs, no 3-acetyl-DON, DON, DOM, α-/β-ZOL, T-2 or HT-2 could be detected. Quantifiable results were only obtained for ENN B, ENN B1 en BEA with maximum concentrations of 15 µg/kg, 2.5 µg/kg and 65 µg/kg, respectively. Plateau levels were reached within 5 to 6 days. Nine to ten days after providing blank feed again, eggs were free of residues. Carry over values were 0.10 %, 0.05 % en 0.44 % for ENN B, ENN B1 en BEA, respectively.

As for the presence of these mycotoxins in commercially available samples, a limited monitoring study was performed in which 30 egg samples (organic, free range, floor housing and cage), 20 poultry meat samples (conventional and organic), 16 pork livers and 10 pork meat samples were examined. All samples were analysed with validated LC-MS/MS methods but none of the mycotoxins studied seemed to be present in the samples.

This study was funded by the Federal Public Service of Health, Food Chain Safety and Environment (contract RF 09/6211).
IMPACT ON ANIMAL AND HUMAN HEALTH – L30

New results for ochratoxin A analysis in infants’ urines

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Ochratoxin A (OTA) is a nephrotoxic mycotoxin contaminant with a widespread occurrence in food commodities, and also found in human biological samples (including breast milk) collected in different geographical areas and countries [1, 2]. As newborns and infants may be more susceptible to adverse effects of toxic contaminants than adults, investigating the exposure of this subgroup with adequate (non-invasive) biomonitoring methods is of importance. Recently, we have shown that OTA concentrations measured in urine samples reflect rather well the dietary mycotoxin intake of infants as calculated from concurrent analysis of breast milk samples [3, 4]. This study in Chilean mother-child pairs demonstrates that OTA concentration in urine is a suitable and valid ‘biomarker of exposure’ for infants.

The thereby validated method for OTA biomonitoring in urine has now been applied to explore mycotoxin exposure in additional cohorts of infants. OTA was determined in urines of Turkish infants (less than 6 months of age) by an established method which involves enzymatic hydrolysis (β-glucuronidase/sulfatase) prior to liquid-liquid extraction with chloroform and LC-MS/MS analysis [3]. OTA was detected (LOD 20 ng/L) in >60% of the urines (17/28); the average concentration was 442 ± 502 ng/L (max. 1360 ng/L). In urines of Chilean infants OTA was detected in >75% of the samples (30/39); the average concentration was 76 ± 100 ng/L (max. 433 ng/L). These values (or the creatinine adjusted OTA urine concentrations) indicate a higher mycotoxin exposure in the Turkish infant cohort compared to the Chilean infant cohort. An additional analysis of OTA metabolites revealed another interesting difference: OTA conjugates (glucuronide/sulfate) were major metabolites in urines of some highly exposed Turkish infants, but not in Chilean babies, indicating that OTA-conjugation may represent an important metabolic pathway at high exposure levels. Further analysis of urine samples from German infants is underway.

References
IMPACT ON ANIMAL AND HUMAN HEALTH – L30
New results for ochratoxin A analysis in infants’ urines
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References

REDUCTION AND PREVENTION – L31
Microbes as bio-control agents against Fusarium pathogens and mycotoxins
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Bio-control agents are relatively safe to the environment, and they have received considerable attention regarding screening strategy and controlling mechanism. Microbes isolated from various ecosystems in China were screened based on their antagonistic effects against Fusarium pathogens and detoxification activity to deoxynivalenol (DON), the predominant Fusarium mycotoxin contaminating cereal grains. Different bacterial species from scabby wheat spike, soil and deep sea have been identified to inhibit Fusarium pathogens in vitro and in planta, and their antagonistic metabolites have been chemically and molecularly characterized. Strains capable of detoxifying DON have been isolated and the detoxified mycotoxin-derived compounds have been analyzed by LC-MS-MS and NMR to reveal their chemical structures. The isolated microbes may be used as basis to develop environment-safe agents to control Fusarium diseases in field and to reduce mycotoxin load in agriculture samples.
REDUCTION AND PREVENTION – L32

Isolation of Rhodococcus erythropolis MTHt3 and cloning of ergA for ergopeptine hydrolysis and ergB for ergine deamination

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Ergot alkaloids, which are produced by members of the fungal orders Hypocreales and Eurotiales, occur worldwide in cereals and grasses. After a long history as cause of epidemics in humans, nowadays intoxication occurs predominantly in livestock after consumption of endophyte infected grasses and leads to great economic losses. Diverse management strategies on the fields pasture management, animal management, and feed treatments have been established. Degrading microorganisms or enzymes, which can be applied as feed enzymes and are active in the gastrointestinal tract, may be a technological solution to ameliorate the problem.

The objectives of this research were to isolate ergot alkaloid-degrading microorganisms and to identify the responsible enzymes. Strain MTHt3 was isolated from soil and identified by phylogenetic analysis based on 16S rRNA as member of the species Rhodococcus erythropolis. The strain was capable of converting all tested ergopeptines (ergotamine, ergovaline, ergocryptine, ergocristine, ergocornine, and ergosine) to ergine and further to lysergic acid. Characterisation of the strain showed that conversion of ergopeptines to ergine occurred in a broad pH and temperature range whereas deamination of ergine to lysergic acid was strongly influenced by pH and temperature. The enzyme responsible for conversion of ergopeptines to ergine (ErgA) was purified by ion-exchange chromatography and hydrophobic interaction chromatography and peptide sequences were determined. The peptide sequences were matched to a gene on the previously identified linear R. erythropolis megaplasmid pMTHL1, which was found by bioinformatic comparison of the R. erythropolis MTHt3 whole genome sequence and confirmed by pulsed-field gel electrophoresis. The whole genome sequence was obtained by 454 de novo sequencing. ErgA was identified as member of the alpha/beta-hydrolase fold superfamily. The amidase ErgB hydrolyses ergine to lysergic acid. Heterologous expression in Escherichia coli BL21(DE3) confirmed the activity of both enzymes. Lysergic acid has lower vasoconstrictive activity compared to ergopeptines and simple lysergic acid amides (Foote et al. 2011). Hence, metabolisation of ergot alkaloids to lysergic acid by R. erythropolis MTHt3 or the enzymes ErgA and ErgB may reduce toxicity. The enzymes may have potential for application as feed additive to reduce the effects of ergot alkaloid-contaminated fodder on animals.

References
REDUCTION AND PREVENTION – L33


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Mycotoxins are wide spread toxins synthesized by fungi contaminating several crops. They are responsible of mycotoxicosis in farm animals. Some polysaccharides from yeast cell wall (3-beta glucane and D-mannan) are efficient adsorbents of mycotoxins. The main objective of this paper is to optimize a standard protocol to assess the efficacy of some mycotoxin adsorbent materials based on their ability in binding zearalenone (ZEA) or ochratoxin A (OTA) or aflatoxin B1 (AFB1) in controlled in vitro conditions. The goal is to define general in vitro parameters (adsorption equilibrium time, pH, mycotoxin concentrations) for the determination of binding efficacy of mycotoxin adsorbents, which can be used to calculate the relevant equilibrium adsorption constants (i.e. binding capacity and affinity). For this purpose, we have analysed the kinetics of binding of mycotoxins on yeast cell wall. Three pHs have been tested: pH 3, pH 5 and pH 7. The percentage of adsorption has been evaluated by quantification of residual mycotoxin in supernatant and amount of mycotoxin adsorbed in residue. Incubation of yeast cell wall in presence of mycotoxins solved in buffer, lead to artefactual adsorption when analysis was based only on residual mycotoxin in supernatant. The decrease of mycotoxins in the supernatant was not correlated to the amount of mycotoxins found in the residue. For this reason we modified the conditions of incubation. Yeast cell wall (5mg) was pre-incubated in buffer (990 µl) at 37°C during 5 min and then 10µl of methanolic solution of mycotoxin were added. After incubation (15-90 min), the solution was centrifuged, and the amount of mycotoxins were analysed both in supernatant and in residue. A plateau of binding (mycotoxin/yeast) is reached after 15 minutes of incubation whatever the mycotoxins tested. The best adsorption of ZEA was obtained at pH 5 (75%), whereas at pH 3 and 7 the maximum of adsorption was 60%. OTA was significantly adsorbed only at pH 3 (50%). AFB1 was only adsorbed to a limited extent at pH 3 (20-30%). Eight products were tested (yeast cell wall or inactivated yeast). The described experimental protocol based on in vitro tests provided reliable isotherms for each mycotoxin. The most suitable models were the Hill model for ZEA, the Langmuir model for AFB1, and the Freundlich model for OTA. From these models, original mathematical affinity criteria were defined to quantify the product adsorption performances for each mycotoxin (Joannis-Cassan et al, 2011).

References
TOXICOLOGY – L34

**New insights into mycotoxin mixtures: the toxicity of low doses of Type B trichothecenes against intestinal epithelial cells is synergistic**

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Deoxynivalenol (DON) is the most prevalent trichothecene mycotoxin in crops in Europe and North America. DON is often present with other type B trichothecenes such as 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON), nivalenol (NIV) and fusarenon-X (FX). Although cytotoxic effects of individual mycotoxins have been widely studied, data on combined toxic effects of mycotoxins are limited. The aim of this study was to assess interactions that occur in situations of co-exposure to type B trichothecenes. Proliferating Caco-2 cells were exposed to increasing doses of type B trichothecenes, alone or in binary or ternary mixture. MTT and Neutral red uptake, respectively linked to mitochondrial and lysosomal integrities, were used for measurement of intestinal epithelial cell viability. The five tested mycotoxins had a dose-dependent effect on proliferating enterocytes and could be classified in increasing order of toxicity: 3-ADON<15-ADON≈DON<NIV<FX. Binary or ternary mixtures also showed a dose-dependent effect. At low concentration (cytotoxic effect between 10 and 30-40%), mycotoxin combinations presented a synergistic effect. At higher concentration (cytotoxic effect around 50%), mycotoxin combinations present an additive or nearly additive effect. These results suggest that the simultaneous presence of low doses of mycotoxins in food commodities and diet may induce more toxicity than that can be predicted from the mycotoxins alone. With regard to the concentrations of mycotoxins consumers are commonly exposed to, the later observation is of highly biological relevance.
Molds of the genus *Alternaria* are a common cause of spoilage in a wide variety of crops, including fruits, vegetables, nuts and cereal grains. In epidemiological studies, a high incidence of esophageal cancer was associated with high levels of *A. alternata* contamination in grain (Liu et al., 1992). *Alternaria* fungi produce numerous mycotoxins including tenuazonic acid, alternariol (AOH), alternariol monomethylether (AME) and altenuene, which have been studied in some detail. Little effort was made this far to examine the toxicological potential of the perylene quinone-type toxins such as altertotoxin (ATX) I, II, III and stemphytotoxin III. Previous studies of ATX I, II, III and stemphytotoxin III in *Salmonella typhimurium* strains have shown that their mutagenic activity increased with the number of epoxide groups in the molecule (Stack and Prival, 1986; Davis and Stack, 1991). The results for the mutagenicity of AOH and AME in *S. typhimurium* are contradictory, but both toxins exhibit a weak mutagenicity at the hypoxanthine-guanine phosphoribosyltransferase (HPRT) gene locus in Chinese hamster V79 cells (Brugger et al., 2006, Fleck et al., 2012).

The aim of the present study was to further investigate the toxicological potential of ATX II, because only few data are available and its risk for public health could not yet be assessed. ATX II is not commercially available but produced by various *Alternaria* fungi. For this reason it was isolated from *Alternaria alternata* and verified by its UV spectrum and by NMR spectroscopy. As toxicological endpoints we examined its cytotoxicity, metabolism, DNA strand breaking activity using the alkaline unwinding method, and its mutagenicity at the HPRT gene locus in V79 cells.

ATX II is a potent mutagen in V79 cells, inducing a concentration-dependent increase of mutations at the HPRT gene locus (Fleck et al., 2012). It is about 50-fold stronger than AOH and AME and therefore approximately as mutagenic as the established mutant 4-nitroquinoline-N-oxide. In contrast to AOH and AME, which lead to a cell cycle arrest in G2/M phase, ATX II does not affect the cell cycle distribution at mutagenic concentrations. DNA strand breaks are also significantly increased by ATX II in V79 cells again exceeding the potency of AOH and AME by a factor of 20 and 5, respectively (Fleck et al., 2012).

The high mutagenic and genotoxic activities of perylene quinone mycotoxins with an epoxide group such as ATX II, ATX III and stemphytotoxin III raise the question whether these *Alternaria* toxins possess a risk for public health and warrant research on the occurrence of these mycotoxins in food and feed.

**References**


Genotoxic properties of Alternariol and its phase I metabolite 4-OH-Alternariol

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Fungi of the genus Alternaria are widespread contaminants and relevant to the spoilage of food and feed ¹. Especially alternariol (AOH), one of the major toxins of Alternaria alternata with respect to quantity is known to possess genotoxic properties thus representing a potential risk for human health. Recently we demonstrated that AOH affects the cellular redox status of cells, but without induction of oxidative DNA damage ². AOH has been reported to represent a substrate for cytochrome P450 (CYP) enzymes in phase I metabolism, generating hydroxylated metabolites with catecholic structure ³-⁴. However, the toxicological relevance of these metabolites has not been elucidated yet.

In the present study we compared the genotoxic properties of the phase I metabolite 4-hydroxy-altanariol (4-OH-AOH) to the parent compound. In human esophageal cells (KYSE510) we investigated the impact of hydroxylation on cytotoxicity, the onset of oxidative stress, topoisomerase poisoning and genotoxicity. In the comet assay enhanced levels of DNA damage was observed after incubation of KYSE510 cells for 1 h with 4-OH-AOH compared to the parent compound. Moreover 4-OH-AOH was found to induce the formation of fpg-sensitive sites which was negligible after incubation with AOH. These findings might be linked to the catecholic structure, potentially allowing redoxcycling and therefore the formation of reactive oxygen species (ROS). To exclude artifacts, cytotoxicity was determined by WST after 24 h of incubation. For AOH, a decrease of 43 % in mitochondrial activity was detected at the highest concentration of 50 µM and 45 % for 4-OH-AOH.

We recently identified AOH as a topoisomerase inhibiting compound ⁵, stabilizing topoisomerase II-DNA-intermediates. As a consequence, DNA double strand breaks might result from the collision of the DNA-topoisomerase complex with the replication fork ⁶. In the decatenation assay, a cell-free test system, 4-OH-AOH exhibited higher potency to suppress topoisomerase II activity compared to the parent compound. Currently studies are in progress to investigate whether this enhanced potency towards topoisomerase II is also reflected on the cellular level or compensated e.g. by changes in pharmacokinetics due to the additional hydroxyl group.

Taken together, in KYSE510 esophagus cells the hydroxylation in 4-position of AOH is associated with an increase of DNA strand breaking properties and the induction of fpg-sensitive sited, indicative for enhanced oxidative DNA-damage. Also a higher potency for the inhibition of topoisomerase II activity might contribute to the DNA-damaging effects in the comet assay. Thus, on the basis of these preliminary data, hydroxylation at 4-position of AOH appears to represent a toxifying metabolic step.

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In medicinal chemistry an integrated approach of docking simulation and rescoring procedure is a well known technique to discover and design new lead compounds. As well, the same tools can be used to predict the interaction between mycotoxins (and any other kind of small molecules) and targets of interest. The endocrine disrupting behaviour can be tightly related to the capability of small molecules to competitively bind the ligand binding pocket (LBP) of estrogen receptors (ERs) and to stabilize at least one of the functionally active conformations of the ligand binding domain (LBD).

Our previous works demonstrate that the study on the interactions between unexpected molecules and alpha isoform of estrogen receptors could be also a valuable choice to discover new hypothetical xenoestrogens.

Herein, we report how in silico approach can be applied to food safety evaluating the estrogenic activity of some mycotoxins and their derivatives. Zearalenone group, afla- and ochratoxins, trichotheecenes, and alternaria family were subjected to docking simulation within both human isoforms of ERs. In order to forecast the LBD-dependent agonistic activity, the interaction within the agonist topology of LBD has been predicted.

Moreover, the interaction between considered compounds and other proteins of relevant bioactivity are also been computed.

As their cognate canonical molecular “wet” techniques, these “dry” methodologies can be used to investigate potential xenoestrogenic role from a molecular point of view, but saving time and money.

References
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Abstracts of posters
DETECTION – P1

Quantitative determination of the *Fusarium* mycotoxins beauvericin, enniatin A, A1, B and B1 in pig plasma using high performance liquid chromatography-tandem mass spectrometry


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Beauvericin (BEA) and enniatins (ENNs) are minor *Fusarium* mycotoxins frequently contaminating different commodities in moderate regions. They are of importance due to their ionophoric properties leading to altered membrane potential and cytotoxic effects *in vitro*. Up to date, no data are available on their *in vivo* toxicity, toxicokinetics nor animal exposure. Further research is therefore needed, however this requires sensitive methods for quantification of those toxins in animal tissues and body fluids.

The goal of present study was to develop a sensitive and reliable method for detection and quantification of BEA, enniatin (ENN) A, A1, B and B1 in pig plasma using liquid chromatography combined with heated electrospray ionization tandem mass spectrometry. Sample clean-up consisted of a deproteinization step using acetonitrile, followed by evaporation of the supernatant and resuspension of the dry residue in acetonitrile/water (80/20, v/v). The chromatographic system consisted of a quaternary, low-pressure mixing pump with vacuum degassing connected to an autosampler with temperature controlled tray and column oven. Chromatographic separation was achieved on a Hypersil Gold® column (50 mm x 2.1 mm i.d., dp: 1.9 µm) in combination with a guard column of the same type (10 mm x 2.1 mm i.d., dp: 3 µm).

Mobile phase A consisted of 0.1% glacial acetic acid in water whereas mobile phase B was acetonitrile (ACN). Following gradient elution program was run: 0–0.5 min (70% A, 30% B), 0.5–2.5 min (linear gradient to 20% A), 2.5–8.5 min (20% A, 80% B), 8.5–10.0 min (linear gradient to 70% A), 10.0–12.0 min (70% A, 30% B). The LC column effluent was interfaced to a triple quadrupole mass spectrometer. For each compound, the two most intense product ions of the precursor ion were monitored in the SRM mode for quantification and identification, respectively. The method was in-house validated as described by De Baere et al. (2011): matrix-matched calibration graphs were prepared for all compounds and correlation and goodness-of-fit coefficients ranged between 0.9980 and 0.9995 and between 5.2% and 11.3%, respectively. The within- and between-run precision and accuracy were evaluated and the results fell within the ranges specified by the EC (2002/657/EC). The limits of quantification were 0.1 ng/mL for ENN A and A1 and 0.2 ng/mL for BEA, ENN B and B1, whereas limits of detection were ≤ 10 pg/mL for all analytes. The method has been applied for the analysis of real plasma samples from one pig that received an oral bolus (0.05 mg/kg BW) of the investigated mycotoxins. At the applied dosage, the results indicated the suitability of the method for use in toxicokinetic studies with ENNs.

Acknowledgments
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References
DETECTION – P2

Development and validation of an LC-MS/MS method for the simultaneous determination of free and conjugated Alternaria toxins in cereal-based foodstuffs

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Alternaria is a common genus of fungi, which contains numerous species that can contaminate a wide variety of crops in the field and cause post-harvest decay of various fruits, grains, and vegetables. In addition to causing economic losses, mycotoxins are formed by Alternaria spp. under certain conditions. Due to the possible risk for public health related to the presence of Alternaria toxins in food, the European Food Safety Authority (EFSA) stipulates that additional quantitative occurrence data is urgently needed to refine exposure assessment. Furthermore, Alternaria toxins can as other xenobiotics partly be metabolised, which may lead to the formation of conjugated metabolites in plants. These modified or "masked" mycotoxins are of human health concern as they probably can release their native precursors after (enzymatic) hydrolysis in the digestive tract of organisms. Therefore, within this research, an LC-MS/MS method for the simultaneous determination of free (AOH, AME, ALT, TeA, TEN & ATX I) and conjugated (AOH-3-sulphate, AME-3-sulphate, AOH-3-glucoside, AME-3-glucoside) Alternaria toxins in cereal products (rice and oat flakes) was developed. Extraction was performed with acetonitrile/water/acetic acid (79/20/1, v/v/v) followed by a hexane defatting step. After filtration, the extract was evaporated and the residue was reconstituted in mobile phase for injection. The mobile phase, which consisted of a mixture of acetonitrile and water, was adjusted to pH 3 with glacial acetic acid. The method, applying isotopically labelled internal standards, allowed for the simultaneous determination of 10 Alternaria toxins in a one-step chromatographic run using a Waters Acquity UPLC system coupled to a Quattro Premier XE mass spectrometer. The method will be validated for several parameters such as linearity, apparent recovery, limit of detection, limit of quantification, precision, expanded measurement uncertainty and specificity (in agree-ment with the criteria mentioned in Commission Decision No. 2002/657/EC). Subsequently, the described methodology will be used for detection and quantification of free and conjugated Alternaria toxins in commercially available cereal-based foodstuffs.

Acknowledgements
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A new approach for the synthesis of stable isotopically labeled fumonisin B\textsubscript{1} standards and analysis of fumonisins in food

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Fumonisins are commonly occurring mycotoxins mainly produced by fungi of the \textit{Fusarium} genus. Because of potential health risks derived from toxicological effects in animals and epidemiological findings along with the common occurrence the EU has set maximum levels for the most prevalent fumonisins B\textsubscript{1} and B\textsubscript{2} (FB\textsubscript{1} and FB\textsubscript{2}) for maize-based food.

To ensure a constant monitoring of samples in regard to this limits convenient and reliable methods for quantitation are required. The coupling of high performance liquid chromatography with tandem mass spectrometry (HPLC-MS/MS) has become one of the most suitable methods meeting many requirements for the analysis. However, the use of this technique is limited by matrix effects impairing the ionization of the analyte. Stable isotopically labeled standards can be applied to compensate for these effects. Another reason for the increasing demand for labeled standards is the recent trend towards the reduction of sample preparation with “dilute and shoot” approaches where the sample is extracted, diluted and analyzed without further cleanup. For this technique stable isotopically labeled standards can also be used to compensate for losses during sample preparation e.g. irreversible binding to matrix components. Synthetic production of sufficient amounts of these labeled compounds is often highly challenging due to their complex structures.

For a quick and convenient synthesis of a suitable standard we started a new approach which was based on the long known exchange of oxygen atoms in carboxyl groups by \textit{H}_{2}\textsuperscript{18}O [1]. We were able to introduce \textsuperscript{18}O-atoms into the side chains of the FB\textsubscript{1} molecule by acid catalysis [2]. The obtained standard proved to be stable enough in regard to usual solvents and storage temperatures. The synthesized standard was successfully employed in the HPLC-MS/MS analysis of maize products after a “dilute and shoot” preparation with results matching those of other validated methods.

References
DETECTION – P4

Rapid and simultaneous detection of aflatoxin B1, deoxynivalenol and zearalenone by lateral flow immunoassay

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Aflatoxin B1 (AFB1), deoxynivalenol (DON) and zearalenone (ZEN) are most commonly found Aspergillus and Fusarium mycotoxins worldwide and frequently co-occurred in food and feed stuffs. All these toxins are immunotoxic, carcinogenic, hepatotoxic and neurotoxic to human and animal health. Therefore, there is an urgent need to on-site and rapidly monitor their contaminations. For this purpose, a lateral flow immunoassay (LFA) based on competitive format was developed and validated for rapid and simultaneous detection of AFB1, DON and ZEN in cereal grains, based on the availability of three specific monoclonal antibodies against the individual mycotoxins.

The designed LFA strip consisted of four sections as follows: sample pad, conjugate pads, nitrocellulose membrane, and absorbent pad. During the preparation of the strip, the sample pads and conjugate pad were treated with blocking buffer and then dried. Absorbent pads were not treated. The competitors of the mycotoxins, the prepared Antibody-Gold Nanoparticles Probe for the mycotoxins, were dispensed onto the conjugated pads. The detector reagents, (AFB1/DON/ZEN-bovine serum albumin conjugates) were immobilized onto a nitrocellulose membrane at three detection zones to form test lines of T1, T2, and T3, respectively.

The performance of the colloidal gold-based LFA strips was evaluated by visually changes. The mycotoxins concentrations were inversely proportional to the color intensity in the colloidal gold-based LFA systems. With this method, the cutoff values for the three test lines were achieved at 5μg/kg for AFB1, 100μg/kg for DON and 100μg/kg for ZEN, which is lower than the maximum residue levels (MRLs) established for those mycotoxins. The recoveries for these mycotoxins were under examination. In-Parallel LC-MS/MS analysis was also in preparation.

Acknowledgement
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DETECTION – P5

Quantitative and sensitive analysis of 15 mycotoxins in foodstuffs using isotopically labelled internal standards applied to liquid chromatography electrospray ionisation tandem mass spectrometry (LC-ESI-MS/MS)

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Hundreds of mycotoxins have already been identified. They can occur in a very broad range of crops and foodstuffs including most notably cereals, nuts, spices or coffee. The current reference methods for their analysis is performed by HPLC coupled to ultraviolet (UV) or fluorescence (FLD) detectors after an immunoaffinity column (IAC) cleanup. HPLC-UV/FLD methods generally exhibit a high sensitivity for a broad range of matrices but are limited to a single compound or to certain classes of mycotoxin. The current trend is the use of LC-MS/MS multiresidue methods allowing a confirmatory detection of mycotoxins. Despite fast and easy protocols have been developed, generic LC-MS/MS methods suffers from a lack of sensitivity especially for Aflatoxins or Ochratoxin A that are regulated by extremely low maximum limits in most notably processed cereal-based foods and baby foods intended for infants and young children.

The aim of this study was to develop a quantitative LC-MS/MS multiresidue method for the analysis of 15 mycotoxins in a broad range of matrices including: corn, cocoa, sunflower oil, paprika, infant formula, green coffee and peanuts. Currently EC-regulated mycotoxins (aflatoxins B1, B2, G1, G2 and M1; fumonisins B1 and B2; ochratoxin A; deoxynivalenol and zearalenone) were surveyed as well as T2, HT-2, nivalenol, and 3- and 15-acetyldeoxynivalenol.

Positive identification of mycotoxins by LC-MS/MS in the sample was conducted according to the confirmation criteria defined in EU Commission Decision 2002/657/EC while quantification was performed by the isotopic dilution approach using fully 13C-labeled mycotoxins as internal standard (IS). The extraction procedure was based on the QuEChERS (acronym of Quick, Easy, Cheap, Effective, Rugged and Safe) protocol, generally applicable to all analytes in cereals, oil, nuts and infant formula. In the particular case of AFLAs and OTA in baby-foods or in difficult matrices such as cocoa, green coffee, nuts, spices and infant formula, the extract was submitted to a parallel IAC clean-up.

The extraction procedure showed good performances for linearity [(r)² > 0.99], recovery [70-120%] and precision [relative standard deviation for repeatability (RSDr) and intermediate reproducibility (RSDa) < 20%), thus fulfilling the EU requirements.

Details on sample clean-up and performance characteristics will be presented.
DETECTION – P6

Rapid biosensor for the detection of mycotoxin in wheat – the MYCOHUNT system


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Most mycotoxins are known to contaminate hazardously crops and consequently animal feeds and food products, causing significant economic losses associated with their impact on animal and human health, animal productivity as well as domestic and international trading. The European Union is setting stricter and stricter limits of mycotoxin concentrations in grains. Deoxynivalenol (DON) is one of the mycotoxins that are formed by a number of species of Fusarium, it is often formed before harvest when crops are invaded by certain species such as F. graminearum and F. culmorum.

Mycohunt project targets the development of a new system incorporating a wheat dust sampling technique guaranteeing a 90% bulk transparency to detect the infection of DON in wheat grains. This MYCOHUNT system incorporates (i) a wheat dust sampling unit and (ii) an immunosensor unit where DON infection is detected by the application of immunosensor with DON-selective antibodies. The system is equipped with a user-friendly computer based control and monitoring unit. The system was fully designed based on the specific needs of grain producers, traders and other relevant players of the sector.

The concept of testing DON from wheat dust has been proven via defining the correlation between DON found in wheat grain samples and in wheat dust samples. The results of dust showed lower level of uncertainty errors than the results obtained when measuring DON in wheat grains. DON selective antibodies have been developed to be used in the immunosensor unit and a specific assay has been developed to obtain the immunosensor characterization. The components of the prototype have been constructed and integrated. The physical realization and the integration of the complete system including the sampling unit, the sensor unit and their control system have been installed to perform field tests during wheat harvesting and storing.

In the future, the MYCOHUNT system can be adapted for the detection of other mycotoxins in wheat or in other crop types such as maize.

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DETECTION – P7

Comparison of different cell lysis methods and ready-to-use kits for DNA-isolation from vegetative spores of certain food-spoiling moulds

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The biomolecular proof of moulds in raw materials and processed food has recently become an important issue (Bleve et al. 2003). Invisible spores might be reactivated during storage or processing and form a mycelium or even produce mycotoxins, which consequently cause damage to product and consumer (Huber et al. 2009). Therefore, a punctual proof of the presence of spores by methods based on DNA-detection is useful. Since spores are extremely resistant, their DNA is difficult to isolate (Saß et al. 2005). Several approaches concerning the DNA isolation have already been described (e.g. Cenis et al. 1992; Motkova 2011; Saß et al. 2005). In this study, vegetative spores are rinsed from overgrown agar plates, counted and adjusted to certain concentrations and treated by three different methods in order to break up their cell walls. By centrifugation, the spores sink to the ground of the sample tube for separation. Shredding with the help of zirconium beads and a swing mill as well as ultrasonic waves of a sonotrode are tested to break up to the cell wall and lay open the spore-DNA. Following four different commercially available DNA-extraction-kits and one kit intended for research only are applied to gain the DNA. They are based on diverse principals, e.g. bonding of DNA on a column and clean-up by several washing steps, denaturation by enhancing the pH-value, precipitation or enzymatic treatment. The aim of the study is to find out the optimal method for cell disruption and for DNA-extraction of the examined spores. Successfully isolated DNA is proved by Real-time-PCR based on specific primer-probe systems. The comparison of Ct-values serves as appraisal criterion for the respective method. Further evaluation criteria are e.g. costs, preparation time and practicability of the method or purity of the isolated DNA. The purpose of the study is to develop a method for routine analysis in food producing facilities.

References
DETECTION – P8
Development and validation of a multi-mycotoxin UHPLC-MS/MS method for the detection of *Fusarium* mycotoxins in eggs and poultry meat

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Contamination of food and feed stuffs by mycotoxins is a widespread problem and has led to a specific legislation for a number of compounds such as aflatoxins, deoxynivalenol (DON), fumonisins, ochratoxin A, patulin, HT-2 toxin (HT-2), T-2 toxin (T-2) and zearalenone (ZEN). In addition to these rather well-known “traditional” mycotoxins, interest is growing in “emerging” mycotoxins like for example enniatin (ENN) A, A1, B and B1 and beauvericin (BEA). Due to their high stability, carry over of mycotoxins present in contaminated feed may occur into animal-derived products such as eggs or poultry meat. Because more data are needed to make a correct risk evaluation for the consumer, this study focused on developing and validating an ultra-high pressure liquid chromatographic-tandem mass spectrometric (UHPLC-MS/MS) method for both eggs and poultry meat for DON (and metabolites), ENN A, A1, B and B1, BEA, HT-2, T-2, ZEN and α/β-zearalenol (α/β-ZOL).

For both matrices (poultry meat and eggs), acetonitrile (ACN) was chosen as extraction solvent. The extraction procedure itself is based on the method described by Mortier et al. (2005a) for egg and Mortier et al. (2005b) for poultry meat. Chromatographic gradient separation (9.5 min) was performed on an UPLC BEH C18 column (2.1x100 mm, 1.7 µm). The mobile phase consisted of H2O + 0.1% formic acid + 1 mM ammonium acetate (A) and ACN + 0.1% formic acid (B). Mass spectrometric detection was achieved on the Xevo™ TQ mass spectrometer (Waters), switching between ESI+ and ESI- within one analytical run. Both methods (eggs and poultry meat) were validated taking into account the requirements of the Commission Decision 2002/657/EC. The validation parameters considered were linearity (R²), apparent recovery (Ra), repeatability (RSDr), reproducibility (RSDR), specificity, limit of detection (LOD) and quantification (LOQ). The expanded measurement uncertainty (U) was calculated using the equation

\[ U = \sqrt{R^2_{bias} + U_{ref}^2}. \]

Calculations were based on relative peak areas, i.e., the peak area of the compound of interest divided by the peak area of the corresponding internal standard (IS) added to the same sample. Internal standards used were 13C-DON, 13C-HT-2, 13C-T-2 and 13C-ZEN.

As for the validation results, Ra varied between 85% and 113% for eggs and between 81% and 103% for poultry meat. RSDr and RSDR were found to be adequate for the different compounds, though RSDR was rather high for 3Ac-DON. The LOD values for egg and meat were below 2 and 3 µg kg⁻¹, respectively, those for LOQ were below 4 and 6 µg kg⁻¹, respectively. The criteria of linearity (R² ≥0.99) and specificity were fulfilled for all mycotoxins and for both matrices studied. The developed methods can be used to determine the degree of contamination in egg and poultry meat, in order to give a better idea of the possible risk for consumers (see presentation “Detection of Fusarium mycotoxins and carry over to animals and humans: case study poultry”).

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References
DETECTION – P9

Quantum dot-loaded liposomes for on-site determination of aflatoxin M1 in milk


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Aflatoxin M1 (AfM1), generated by hydroxylation of aflatoxin B1 (AfB1) in mammals and subsequently secreted in the milk of lactating cows, is one of the most dangerous pollutants of milk and milk products. Both AfB1 and AfM1 are characterized with carcinogenic and hepatotoxic activity. AfM1 has been classified as a Group 1 carcinogen by the International Agency for Research on Cancer (IARC). The maximum residue limit (MRL) of AFM1 in raw milk, heat-treated milk and milk for the manufacture of milk based products according to European Commission Regulation was set at 0.05 µg kg⁻¹.

Along with standard chromatographic techniques for AfM1 determination, characterized with the opportunity to determine several mycotoxins with relatively high sensitivity, but time-consuming, complicated in realization and required the using of expensive equipment, sensitive on-site tests suitable for the non-laboratory application are becoming more and more popular. Rapid analysis techniques are especially important for analysis of milk products, because their short lifetime demands the no-delay screening. Big variety of rapid immunochemical tests for control of the AfM1 content was described. All of them are appropriate for practical application, but some techniques required to use different kinds of equipment to register an analytical signal (as sensors or ELISA), another ones could not reach the claimed sensitivity (comparable with established EU MRL). Column gel-based immunoassay allows to combine purification, concentration and direct analysis in one step. Replacement of horse radish peroxidase as a label for quantum dots (QD) or QD-loaded liposomes allowed to diminish the analytical procedure, simplify the visual recognition of analytical signal and considerably amplify the sensitivity of test-method.

Novel sensitive column test for AfM1 determination was developed and based on fluorescent determination of the analytical signal. Different immunochemical reagents, QD, conjugation techniques and analysis conditions were compared and estimated. As carriers for antibody immobilization the thin polyethylene frits were chosen. Achieved cut-off value was 0.01 µg kg⁻¹ with a visible and clear contrast in luminescence between positive and negative samples. An intra-laboratory test validation was performed with sterilized milk samples artificially spiked with AfM1 at concentrations less, equal and more than the cut-off level. Performance parameters were calculated based on the summarized data of repeated experiments. Concluding, the rates for false positive and negative results were below 5% (2.6 % and 3.3, respectively) and the specificity and sensitivity rates were in conformity with all parameters set by the Commission Decision 2002/657/EC.
The use of microflow UHPLC in mycotoxin analysis

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Traditionally in mycotoxin screening of food samples, samples are extracted and analysed by LC/MS/MS, usually at LC flow rates which are in excess of 500 µl/min and in combination with high pressures with smaller particle size HPLC columns to maintain sharp peaks and fast chromatography. These flow rates produce fast speeds and excellent peak shapes and results, but have a draw back in that they require higher volumes of organic solvent. The consumption of HPLC organic solvents, such as acetonitrile and methanol, is a growing cost of analysis and its disposal has an environmental impact. Therefore, ways to reduce solvent consumption in pesticide residue testing will be beneficial to the environment and reduce running costs of a testing lab.

Here we present new data using microflow LC (running below 40 µL/min) in combination with a LC-MS/MS method developed on an AB SCIEX QTRAP® system utilizing the Scheduled MRM™ algorithm with the acquisition of MS/MS spectra for compound identification. Initially this approach has been applied to a screen of ergot alkaloids and alfatoxins to show its applicability in food analysis and data presented with compare Microflow LC approach with the traditional LC flow rates.
DETECTION – P10

Detection of Mycotoxins: New Methods and Applications

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Abstracts of posters

DETECTION – P10

The use of microflow UHPLC in mycotoxin analysis

Traditionally in mycotoxin screening of food samples, samples are extracted and analysed by LC/MS/MS, usually at LC flow rates which are in excess of 500 µl/min and in combination with high pressures with smaller particle size HPLC columns to maintain sharp peaks and fast chromatography. These flow rates produce fast speeds and excellent peak shapes and results, but have a drawback in that they require higher volumes of organic solvent. The consumption of HPLC organic solvents, such as acetonitrile and methanol, is a growing cost of analysis and its disposal has an environmental impact. Therefore, ways to reduce solvent consumption in pesticide residue testing will be beneficial to the environment and reduce running costs of a testing lab.

Here we present new data using microflow LC (running below 40 µL/min) in combination with a LC-MS/MS method developed on an AB SCIEX QTRAP® system utilizing the Scheduled MRM™ algorithm with the acquisition of MS/MS spectra for compound identification. Initially this approach has been applied to a screen of ergot alkaloids and aflatoxins to show its applicability in food analysis and data presented with compared Microflow LC approach with the traditional LC flow rates.

DETECTION – P11

Development of a stable isotope dilution LC-MS/MS method for the Alternaria toxins Tentoxin, Dihydrotentoxin and Isotentoxin

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The cyclic tetrapeptide tentoxin (cyclo[N-methyl-L-valyl-L-leucyl-cis-alpha,beta-dehydro-N-methylphenylalanylglycyl], TEN) is a secondary metabolite produced by some Alternaria species. [1] It is considered a phytotoxin and induces species-selective chlorosis. Along with TEN, dihydrotentoxin (DHT) and isotentoxin (isoTEN) were also isolated as metabolites of Alternaria species. Until now, there is limited analytical data of these toxins and, therefore, the corresponding risk assessment is difficult. To investigate the occurrence of these toxins, we developed a stable isotope dilution LC-MS/MS method.

Triply deuterated internal standards were prepared via chemical synthesis,[2, 3], introducing the labels in the last step before cyclization. They were characterized by LC-UV, MS and 1H-NMR.

A series of solutions with constant amounts of internal standard and varying amounts of analyte in molar ratios from 0.1 to 10 was prepared for the calibration curves. Food samples were extracted in acetonitrile-water (84:16, v/v). Clean-up was performed on C18-phenyl column SPE. The three toxins were separated on a BDS C18 HPLC column, detected in the negative ESI mode and quantified by multiple reaction monitoring (MRM).

The response between analyte and internal standard was found to be linear with a coefficient of determination exceeding 0.998 for each of the three toxins. Limits of detection were 0.18, 0.35 and 0.19 µg/kg for TEN, DHT and isoTEN, respectively, for solid food samples. The inter-/intra-day relative standard deviations of the method were below 10 % and the recoveries ranged between 98 and 110 %. The method was applied to 103 food samples including bread, cereals, chips, juice, nuts, oil, sauce, seeds and spices. Of these, 85 % were contaminated with TEN and 55 % were contaminated with DHT, whereas isoTEN was not quantifiable. Maximal concentrations of TEN and DHT were 52.4 and 36.3 µg/kg, respectively, and were both detected in paprika powder.

References

DETECTION – P12

A simple and reliable liquid chromatography-tandem mass spectrometry method for determination of aflatoxin M1 in milk

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Aflatoxins are the most relevant group of mycotoxins potentially impairing the safety of food and feeds. Of particular concern is the AFM1 contamination of animal origin product, the hydroxylated derivative of AFB1 that is formed and excreted in the milk of lactating animals after the ingestion of AFB1 contaminated feed. Indeed, although AFM1 shows a lower toxicity, on account of its probable carcinogenicity (group 2B, IARC 1993) and its frequent occurrence in milk and dairy products, specific regulations for AFM1 in milk and milk products have been set in about 60 countries. In order to allow an extensive and effective control of the occurrence of AFM1 in milk, a number of sensitive and reliable methodologies have been developed. In any case, on account of the interferences due to the high fat and protein content in milk, sample extraction generally results long and tedious, involving several clean-up steps to remove the co-extracted material from the matrix. Thus, the aim of this study is the development of a quick, cheap and accurate protocol for the determination of aflatoxin M1 in milk, by application of a LLE approach followed by LC-ESI-MS/MS analysis.

Liquid-liquid extraction is performed on the defatted milk added with sodium chloride by using ethyl acetate as extraction solvent. Results of the validation study with the proposed chromatographic method are very good in terms of LOQ (15 ng/Kg), accuracy and precision. Mean overall recovery of 96.20 was evaluated on spiked blank milk samples with a confidence interval of 2.86%. The method showed a very good repeatability (mean CV: 2.83%). On 6 different reference materials, the mean overall recovery (n = 24) was 94.9% with a confidence interval of 1.86% and a CV% of 4.54%. The performance of the proposed method was compared with that of the Official ISO Method based on the use of IAC columns: LLE protocol could be considered a valid alternative to the HPLC-IAC. In general it showed in general a better accuracy with a lower data dispersion. Moreover, sample preparation is very simple and straightforward, potentially being applicable as high throughput method which, on account of its simplicity and low cost, may be applied to the analysis of a vast number of samples in the occasion of outbreaks of contamination.
Aflatoxins are toxic metabolites produced by certain strains of the fungi *Aspergillus flavus* and *A. parasiticus* in or on foods and animal feeding stuffs. Aflatoxins have potent carcinogenic effects in susceptible laboratory animals and acute toxicological effects in humans. 18 different types of aflatoxins have been identified although the major members are aflatoxins B$_1$, B$_2$, G$_1$ and G$_2$, and M$_1$ in milk. Aflatoxin B$_1$ is normally predominant in amount, in cultures as well as in food products. The extraction of aflatoxins using immunoaffinity clean up and then detection by HPLC is common practice, and although analyte recoveries are measured regularly by standard addition (spiking), there is no standardisation of the spiking procedure which could potentially have an effect on the measurement of aflatoxin concentrations.

In the UK the Government Chemist is required to act as the national focus of technical appeal in specified areas where there is an actual or potential dispute between food businesses and regulator. Disputes involving aflatoxin contamination in foods or feeds account for a significant percentage of cases carried out by the Government Chemist.

The analytical methods used in this project for the analysis of aflatoxins have been established in LGC for many years, are ISO 17025 accredited, and have been used for the analysis of milk, spices, peanuts and numerous other sample types (matrices). The aim of this project was to investigate the effect, if any, of the spiking time (contact time) on the aflatoxins recovery. The matrices that were studied were peanuts, figs and chillies. Generally it was found that recovery is dependent upon contact time for up to 36h and this effect is statistically significant. However extending the experiment to up to 8 hours didn’t show a statically significant difference.

It is interesting to see that three different matrices which are commonly analysed for aflatoxins behave in a different way when spiked. Although care was taken that the experiment was carried out under strict repeatability conditions and that it was concluded within a reasonable time frame to minimise uncertainty there appears to be a strong interaction between contact time and recovery for figs and peanuts in the first 24h after which recovery seems to be stable for the remaining part of the experiment.
DETECTION – P14

Simple and fast methodology for the determination and identification of multiclass mycotoxins in pseudocereals, spelt and rice samples

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Nowadays the interest and consumption of pseudocereals (buckwheat, quinoa and amaranth) is increasing due to its nutritional properties. Like cereals and oilseed, pseudocereal seeds are susceptible to fungal growth and mycotoxin contamination; however these matrices have received little attention in literature. For these reasons in this work we have considered the proposal of a multimycotoxin method for quality control of these products.

This study also included other cereals such as spelt and different types of rice (white, brown and red). Brown rice is increasingly being chosen by customers because of its health benefits, whereas red rice has been traditionally used in China due to its medicinal properties (anti-hypertensive, anti-diabetic and blood circulation regulator). Nowadays it is an important component of the Chinese diet. Red rice is obtained by the fermentation of normal rice with fungal from genus Monascus. In relation to spelt, although it was about to disappear due to its low yield, now it is increasingly valuable for food product manufacturer and consumers due to its nutritional properties, high resistance in unfavourable environmental factors and lower fertilization requirement compared to wheat. However, to the best of our knowledge, no studies have been carried out on the occurrence of mycotoxins in spelt.

A sensitive, simple and rapid method for the determination and identification of fifteen mycotoxins (aflatoxin B₁, aflatoxin B₂, aflatoxin G₁, aflatoxin G₂, ochratoxin A, fumonisin B₁, fumonisin B₂, nivalenol, deoxynivalenol, fusarenon-X, T-2 and HT-2 toxin, citrinin, sterigmatocystin and zearalenone) in pseudocereals (buckwheat, quinoa and amaranth) has been developed and validated by using ultra high performance liquid chromatography coupled with mass spectrometry (UHPLC-MS/MS) and a sample treatment based on the QuEChERS procedure (1). The method covers all the mycotoxins included in the regulation (EC) 1881/2006 (2), and others considered as hazardous substances by the International Agency for Research on Cancer (3). Matrix-matched calibration curves were established and LODs and LOQs were below the maximum content allowed by EU regulation in cereals. The precision (repeatability and intermediate precision) was estimated, with RSD lower than 12%. Recoveries were between 60.0% and 103.5%, fulfilling the current legislation. Prior to the recovery studies, preliminary analyses of all the selected products were performed in order to check the absence of mycotoxins under study. None of the samples gave positive results, except a red rice sample for AFB₁ (8.3 µg Kg⁻¹). In order to confirm this result, the positive sample was analysed using the standard method based on the application of immunoaffinity columns for cleaning-up, obtaining a relative error of 3.7%, and confirming the trueness of the method.

Acknowledgements
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References
Mycotoxins are toxic natural secondary metabolites produced by several species of fungus, such as for example *Aspergillus* and *Fusarium* species, on agricultural commodities in the field or during the storage. Fumonisins (FUMs) and Deoxynivalenol (DON) are mycotoxins produced by different *Fusarium* species such as *F. graminearum* and *F. moniliforme*. DON is usually found in a large variety of commodities such as corn, wheat, barley and oat and their derived products, while FUMs are more focused on corn and corn-based products. Aflatoxins (AFLs) are produced by *Aspergillus flavus* and *parasiticus* which are able to grow in very different matrices like corn, wheat, (dried) fruits and spices. Those three toxins can have negative impacts on the human and animal health if they are consumed at higher levels than the European regulations (EC No 1881/2006) or at lower levels but in association with other mycotoxins. Indeed, it is now well known that all those toxins are immunotoxic, carcinogenic, hepatotoxic and neurotoxic. Therefore, the monitoring of those toxins in food and feed is primordial. For that purpose, different methods could be used as confirmatory methods such as HPLC and LC-MS or as screening methods like ELISA and lateral flow devices (LFDs). The use of screening methods allows high throughput, cost-efficient analyses and on-site measurements that could be performed by unskilled people. LFDs for mycotoxins detection in food and feed have recently been developed in the last five years. Those rapid tests involve a gold-labelled antibody specific to the target mycotoxin competing with the mycotoxin potentially present in the analysed sample and the same toxin immobilized on the nitrocellulose membrane through a protein conjugate. With such a test, the higher the mycotoxin concentration in the sample, the less intense the test line. The first generation of dipstick tests devoted to the mycotoxins detection was qualitative tests giving a positive or negative response. To date, farmers and grains collectors want first to know if their commodity is contaminated in mycotoxins but also want to know the exact level of this contamination for financial and applications purposes. Therefore, Unisensor has decided to develop a wide range of quantitative lateral flow devices for the most relevant mycotoxins. In this work, we are presenting the new developments of those quantitative LFDs especially for FUMs, DON and AFLs detection in different commodities.

**Fumosensor®, Donsensor® and Aflasensor® Quanti** have been developed for the respective quantification of Fumonisins (B1, B2 and B3), DON and Aflatoxins (B1, B2, G1, G2) in different commodities. Those tests are supposed working with an optical reader (Readsensor®) which translates the test line intensities into mycotoxin concentrations thanks to pre-established calibration curves (varying from lots to lots) saved inside 2D barcodes. Mycotoxins are recovered from the commodity by a simple vortex mixing in alcoholic media during 2 minutes. The dipstick tests are run in only 5 minutes. Quantification ranges for FUMs (200 to 10000 µg/kg), DON (200 to 3000 µg/kg) and for AFLs (2 to 60 µg/kg) have been determined. Those ranges can be further extended by a simple extract dilution. The accuracy of Fumosensor®, Donsensor® and Aflasensor® Quanti has been analyzed by comparing LC-MS results with results issued from dipstick analyses on reference contaminated materials. Coefficients of variation less than 20% have been calculated showing the “fit for purpose” character of these new quantitative dipstick tests as screening techniques.
DETECTION – P16

Development of the first quantitative multiplex dipstick for the simultaneous detection of DON and ZON mycotoxins in grains


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Mycotoxins are secondary metabolites that are produced by fungi in favourable climatic conditions. Among those toxins, zearalenone (ZON) and deoxynivalenol (DON) are two major toxins commonly associated to cereal products such as wheat, maize, oat and barley and feed such as DDGS and silage#. They are mainly both produced by Fusarium graminearum fungus explaining their co-occurrence in certain type of food and feed. DON and ZON are recognized having respectively neurotoxic and oestrogenic effects on the human health in addition of their well-known immuno-suppressive property. Due to their hazardous consequences to human health, their presence in food has been regulated through EU regulations (EC No 1881/2006). Their occurrence in cereals and in cereal-based products is usually monitored with confirmatory methods such as HPLC or LC-MS. Those methods are reliable and give accurate quantitative results. Anyway, they are very expensive, they need of skilled people and do not allow field measurements. The necessity for farmers and grain collectors in having fast detection methods being accurate, inexpensive and allowing on-site measurements has prompted us to develop lateral flow devices (LFDs) for the monitoring of mycotoxins in food and feed. Most of the commercialized LFDs give a positive/negative type response and only target one single toxin. People who are interested in the detection of several mycotoxins are therefore forced to run a number of tests corresponding to the number of toxins they are interested in, representing a lost of time and money. In this work, we are presenting the very first development in the LFDs market of a quantitative dipstick test allowing the simultaneous detection of DON and ZON. This new development represents for the end-user a time saving since only one extraction experiment is needed to recover both toxins and only one dipstick test gives a numerical value of the contamination on each mycotoxin.

The 2-Mycosensor® dipstick test is a multiplex dipstick test developed by Unisensor for the simultaneous quantification of DON and ZON mycotoxins in raw cereals and feed. This test is made of two test lines respectively for DON and ZON and one control line. Each test line is analyzed by an optical reader (Readsensor®) and translated into a DON and ZON concentration thanks to pre-established calibration curves saved in this reader. DON and ZON are both extracted with an alcoholic medium for 2 minutes with a vortex mixer. Recoveries higher than 80% have been measured for all matrices. The dipstick test only takes 5 minutes to get the concentration of DON and ZON present in the sample. Six dipstick tests can be run at the same time, making possible the analysis of DON and ZON concentrations contained into 6 samples in less than 20 minutes, extraction time included. Limits of quantification of 200 µg/kg for DON and 50 µg/kg for ZON have been determined and quantification ranges going from 200 to 3000 µg/kg for DON and from 50 to 750 µg/kg for ZON have been measured without any further sample extract dilution. Those two quantification ranges can be extended by a simple extract dilution. The accuracy of the 2-Mycosensor method has been analyzed by comparing LC-MS results with results issued of the 2-Mycosensor on reference contaminated materials. Coefficients of variation less than 20% have been calculated showing the “fit for purpose” character of this new multiplex quantitative dipstick test as a screening technique.

References
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**DETECTION – P17**

**Determination of the *Alternaria* mycotoxins alternariol and alternariol monomethyl ether in animal plasma using LC-MS/MS**

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*Alternaria* mycotoxins are produced by fungi of the genus *Alternaria*. Several of these mycotoxins, including alternariol (AOH) and alternariol monomethyl ether (AME), have been detected in feed. Monbaliu et al. (2010) found 3/82 feed samples positive for AOH (mean concentration 20.3 µg/kg, range 17-25 µg/kg) and 1/82 feed samples positive for AME at a concentration of 19 µg/kg. *In vitro* studies clearly demonstrate the cytotoxic, mutagenic and elastogenic effects of AOH and AME (Fehr et al., 2009, Fleck et al., 2012). Fleck et al. (2012) showed a cytotoxic and mutagenic effect of these toxins in Chinese hamster V79 cells, starting at 10µM AOH and 20µM AME. Fehr et al. (2009) found an increase in DNA strand breaks after 1h incubation with AOH and AME at concentrations ≥ 1µM in human carcinoma cells. Up to date, there are no *in vivo* genotoxicity or carcinogenicity data available for AOH and AME.

According to the European Food Safety Authority (EFSA), the database concerning toxicological effects of *Alternaria* toxins in experimental animals and humans is currently too limited to be used as a basis for identification of reference points for different toxicological effects of these compounds. EFSA recommends more studies regarding the toxicity and toxicokinetics of AOH and AME (EFSA, 2011). To perform these toxicokinetic studies, sensitive methods are needed to detect and quantify these toxins in animal tissue and body fluids, such as plasma.

Although several analytical methods have been developed for the quantitative determination of mycotoxins in feed (Monbaliu et al., 2010), to our knowledge, no method has been described for the determination of *Alternaria* mycotoxins in plasma, enabling a thorough investigation of the *in vivo* toxicity. Therefore, our goal is to develop a sensitive and specific analytical method for the quantitative determination of AHO and AME in plasma using LC-MS/MS. The method, validated in accordance with the EU Directive 2002/657/EC, will be presented at the congress.

**References**

EFSA on Contaminants in the Food Chain (CONTAM), 2011. Scientific Opinion on the risks for animal and public health related to the presence of *Alternaria* toxins in feed and food. EFSA Journal, 9, 2407.


DETECTION – P18

High specific determination of *Fusarium* mycotoxins in wheat and maize using online sample preparation - high resolution mass spectrometry coupling

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Mycotoxin analysis is still one of the highest importance in food testing laboratories and continuously challenging due to the increasing number of target compounds and their different chemical properties. Recently SPE and immune-affinity columns got widely used providing specificity, high recovery and clean extracts, but they are still rather expensive and time consuming. Consequently, establishment of a cheap and rapid multi-analyte method still remains a demand.

Liquid phase online sample preparation techniques possess lot of benefits over offline sample preparation techniques and are potential options to improve method effectiveness. Through online coupling, advantages of solid phase extraction and direct injection can be combined. The resulted closed system setup and elimination of the most significant error source (human factor) from the sample manipulation enable much faster sample preparation, lower operational costs and simultaneously increase both method repeatability and reproducibility values.

On-line coupling of Turbulent flow (TurboFlow) chromatography (TFC) and conventional HPLC or (U)HPLC techniques (TLC) is one possible option to perform on-line sample preparation method. The effective separation of matrix and usually smaller target compounds based on the different diffusion coefficient values results less matrix load on the chromatographic column and consequently less matrix effect and contamination in the detector. The additional enrichment capabilities of the TFC columns contribute to improved method performance in terms of higher method sensitivity and selectivity values. Further increase in method selectivity and sensitivity can be achieved by application of high resolution-accurate mass spectrometer as detector. Orbitrap technology with ultra-high mass resolution of R=100,000 and additional high energy collision dissociation fragmentation was applied providing more confident and accurate measurement results.

This presentation reports in details over a novel innovative and very promising TLC-HRAM/MS coupled method for the determination of six different mycotoxins in wheat, maize and animal feed matrices [1]. Results of in-house validation including external Fapas proficiency test results ([z]<1 score values) and comparison of established method performance parameters to the current European regulatory limits and requirements will be discussed. The presentation also provides a comparison on pros and contras for both new developed and conventional analytical methods with special focus on method performance parameters like matrix effects, sensitivity and repeatability values.

References
DETECTION – P19

Development of a *Botrytis* specific immunosensor towards using PCR species identification

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Internal defects of onions, particularly neck rot, caused by *Botrytis* species result in considerable losses in field and during storage, due to poor quality and substandard produce. A rapid, sensitive and reliable method to assess fungal pathogen load of infected onions could help to prescribe an effective curing regime, thus reducing postharvest loss. This would subsequently increase retailer and consumer confidence. Furthermore, growers would have the ability to predict and manage the storage potential of their crops.

An enhanced sensitivity electrochemical immunosensor based on a screen-printed gold working electrode (SPGE), with onboard carbon counter and Ag/AgCl pseudo-reference electrode was developed for the detection and quantification of *Botrytis* species. A monoclonal antibody against *Botrytis* was immobilised on the surface of the gold working electrode using the appropriate surface chemistry for the immuno and DNA sensors respectively. A direct sandwich enzyme-linked immunosorbent assays (ELISA) format was then developed using polyclonal antibodies conjugated to the electroactive enzyme horseradish peroxidase (HRP) and to gold nanoparticles for enhanced sensitivity. Electrochemical measurements were then conducted using 3,3’,5,5’-Tetramethylbenzidine dihydrochloride (TMB)/H2O2 as the enzyme mediator/substrate system. Initial tests of the biosensor demonstrated its capability to detect all three species considered as the primary cause of neck rot (B. *allii*, B. *aclada* and B. *byssoidea*), as well as B. *cinerea*, all within a detection limit as low as 0.058 µg ml–1 fungal mycelium.

The second stage of the development combines the biosensor with DNA testing procedures in order to detect and separate the *Botrytis* species. Genetic differentiation between the *Botrytis* species associated with Neck Rot was performed using PCR amplification and restriction fragments length polymorphism (RFLP) differentiation. Real time PCR was used to identify the pathogen DNA with primers specific to *Botrytis* species causing neck rot. A DNA based procedure is now being optimised to combine the biosensor with PCR techniques for early detection and identification of *Botrytis* in the field.
Opportunities and drawbacks of microLC-MS/MS for the use in multi-mycotoxin analysis

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Recent Studies report a growing interest in LC-MS/MS based methods for the simultaneous detection and quantitation of mycotoxins. Methods have been published for several matrices, including food, feed and physiological samples of human and animal origin.

Mycotoxins represent an inhomogeneous group of compounds with regard to their chemical properties. Chromatographic separation therefore remains one of the major challenges in the development of rugged and exact methods for multi-mycotoxin detection and quantitation. Run times of up to 30 minutes are still a major drawback for the use of current methods in routine applications.

Newly introduced microLC systems are promising faster run times and higher sensitivity through the use of reduced diameter columns. This study compares the performance of conventional flow HPLC-MS/MS multi-mycotoxin methods to microLC-MS/MS couplings.

References
DETECTION – P21

Determination of masked mycotoxin derivatives and metabolites of deoxynivalenol in plasma and cell culture media using LC-MS/MS

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The research towards the toxicity, toxicokinetics and occurrence of deoxynivalenol (and mycotoxins in general) has made significant progress during the last years. As the information and insight on native mycotoxins increases, the focus of this research domain tends to shift towards the gathering of occurrence and toxicity data on masked mycotoxins. These are mycotoxin conjugates or derivatives that are undetectable by conventional methods due to changes in their structural formation and physicochemical properties. Masked mycotoxins can be produced by either fungal (i.a. acetylation) or plant (i.a. glycosylation) metabolism. Additionally, food/feed processing can also play an important role in the production or concentration of these compounds in certain products (1). Conversion of these masked mycotoxins back to their native form by in vivo hydrolysis cannot be excluded. Consequently, this would imply an underestimation of the degree of contamination upon analysis.

The goal of this study was to develop and validate an LC-MS/MS method to determine deoxynivalenol related substances: native deoxynivalenol (DON), deoxynivalenol-3-glucoside (DON3G), 3-acetyl-deoxynivalenol (3ADON), 15-acetyl-deoxynivalenol (15ADON), deepoxy-deoxynivalenol and deoxynivalenol-3-glucuronide (DON3GlcA) in plasma and cell culture media. The developed method is the first in plasma and cell culture media to combine these masked forms and metabolites of DON. Furthermore, it has the unique feature of offering a chromatographic base line separation of the two acetylated isomers rather than working with “selective” mass spectrometric transitions as is often the case (Figure 1).

This method will enable us to investigate the intestinal permeability of these compounds with human (Caco-2) and pig (PICE-12) intestinal epithelial cell lines as well as with intestinal tissue explants in Ussing chambers. In a final stage, in vivo experiments (chicken and pig as target animals due to the high exposure of these species and pig to serve as a human model) will enable us to create a complete and comprehensive overview of the toxicokinetic properties of DON and its masked forms. These toxicokinetic observations together with data on cytotoxicity and intestinal cytokine response should provide an indication whether there is a necessity to include these masked mycotoxins in the current European legislation.

References

Acknowledgement
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DETECTION – P22

Metabolome annotation of *Fusarium graminearum* using stable isotopic labelling and LC-HRMS

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This work presents the workflow for the non-targeted analysis of the extracellular metabolome of *Fusarium graminearum* using stable isotopic labelling (SIL) and liquid chromatography high resolution mass spectrometry (LC-HRMS). *Fusarium* cultivars were separately grown in parallel on natural U-¹³C- and U-¹²C-glucose enriched media and 1:1 (v:v) ratio mixtures of the supernatants were subsequently measured with an LTQ Orbitrap XL. The artificial isotopologue pattern (see Figure 1) derived from the labelling step was used for data evaluation which was performed using an in-house developed software application (Bueschl et al., 2012). This program automatically detects MS signals originating from isotopically labelled metabolite ions allowing a reliable extraction of only those features derived from the measured fungi supernatants.

Figure 2: Isotopologue pattern exemplified on two ion species of a metabolite containing 15 carbon atoms. The mono isotopic ¹²C mass peaks of the protonated and sodium adduct as well as the corresponding ¹³C isotopologue patterns are clearly visible. These two mirror symmetric isotopic distributions are used to distinguish between biological derived and non-relevant information. Additionally, both ion forms ([M+H]+ and [M+Na]+) are assigned to the same metabolite in a non-targeted fashion.

All extracted features have been verified using the Pearson correlation coefficient by comparing and confirming the peak shapes of the U-¹²C- and U-¹³C-metabolite ions, annotated with the number of carbon atoms derived from the labelling process and different features were grouped together in a non-targeted fashion to identify various adduct and in-source fragments of the same metabolite. The presented approach illustrates the complete methodical sequence from cultivation of biological samples to data interpretation. Several hundred metabolite features have been extracted, grouped and partly identified or annotated for *F. graminearum*. Moreover, metabolite production was monitored as a function of time and the biological and technical precision of the presented approach has been evaluated.

References

In farm animals, mycotoxins can cause among others, decreased performance, feed refusal, poor feed conversion, diminished body weight gain, immunosuppression, reproductive disorders and residues in animal food products. Many mycotoxigenic fungi can grow and produce their toxic metabolites under similar conditions. Therefore in animal feed, mycotoxins rarely occur as single contaminants. Apart from that, blends of various raw materials in compound feed can increase the risk of feed contamination with several mycotoxins. Several combinations of mycotoxins may lead to additive or synergistic toxic effects. Although the prevention of mycotoxin contamination in the field is the main goal of agricultural and food industries, under certain environmental conditions, the contamination of various commodities with mycotoxins is unavoidable. By analogy with chromatographic methods, the new trend is to develop fast screening multi-mycotoxin methods. This work is focused on the development and validation of a multiplex flow-through membrane-based assay (MBA) for the simultaneous detection of six mycotoxins in a wide variety of food and feed.

Recently, this research group successfully developed a qualitative MBA for the simultaneous screening of four mycotoxins in maize, peanut cake and cassava. The MBA can be used to simultaneously screen for zearalenone, aflatoxin B1, deoxynivalenol and ochratoxin A at 175 µg/kg, 5 µg/kg, 700 µg/kg and 3 µg/kg as cut-off levels respectively. The MBA was validated within a laboratory setting and also under tropical conditions. The false negative rate following both validation studies was <5%, hence fulfilling the strict regulatory guideline as specified in Commission Decision 2002/657 EC. Further challenges will include broadening the scope of the existing MBA to include other mycotoxins and other food and feed matrices.
DETECTION – P24

Development and validation of a QuEChERS based liquid chromatography tandem mass spectrometry method for the determination of multiple mycotoxins in spices

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A reliable and rapid method for the determination of multiple mycotoxins was developed using a QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) based extraction procedure in highly pigmented and complex spice matrices, namely red chilli (Capsicum annum ssp.), black and white pepper (Piper nigrum ssp.). High-performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) was used for the quantification and confirmation of 17 chemically diversified mycotoxins. Different extraction procedures were studied and optimized in order to obtain better recoveries. Mycotoxins were extracted from the hydrated spices using acidified acetonitrile (1% formic acid), followed by partitioning with NaCl and anhydrous MgSO₄; excluding the use of dispersive-solid phase extraction. Significant matrix effect was compensated using the matrix matched calibration curves. Electrospray ionization at positive mode was applied to simultaneously detect all the mycotoxins in a single run time of 20 min. Multiple reaction monitoring (MRM) mode, choosing at least two abundant fragment ions per analyte was applied. Optimized extraction and instrumental conditions gave coefficients of determination in the range of 0.9844 to 0.9997 for all the analytes. Recoveries (ranging from 75 to 114%) were in accordance with the performance criteria required by the European Commission. Intra-day reproducibility ranged from 4 to 22% for most of the mycotoxins. The limit of quantification ranged from 2.3 to 146 µg kg⁻¹. The validated method was finally applied to screen mycotoxins in ten of each spice matrix. Aflatoxins, ochratoxin, fumonisins, sterigmatocystin and citrinin were among the detected analytes. Positive findings were further confirmed using relative ion intensities. The potentiality of the method to be used for confirmatory purposes according to Commission Decision 2002/657/EC was assessed.

Keywords: Mycotoxins, spices, QuEChERS, LC-MS/MS, red chilli, pepper
DETECTION – P25

Catching toxins with peptides: a preliminary study for OTA complexation

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The employment of synthetic molecules other than antibodies (Abs) as active recognition elements in sensing systems has recently gained interest for research purposes. A wide spectrum of non Ab-based strategies have been developed, including aptamers, molecular imprinted polymers and peptides. Despite several synthetic peptide-based sensors have been designed for metals, the development of peptides with complexation capabilities toward small organic molecules have been only recently investigated. Concerning mycotoxins, approaches based on combinatorial chemistry, in silico de-novo design and computer-aided analysis of natural receptors have been proposed for the development of peptide-based complexing elements for ochratoxin A (OTA).

In order to investigate the peptide-base approach for toxin recognition, we decided to focus our efforts on a previously reported sequence for OTA detection, developed by an in silico de-novo design method. However, two modifications have been introduced: the Cys residue at C-term used for linking the peptide to surfaces has been removed; on the other hand, a Trp residue have been added at the N-term with the intent to analyze the complex in homogeneous systems.

Preliminary fluorescence measurements were performed with the aims to: i) assess variations in OTA fluorescence upon complexation; ii) assess variations in Trp fluorescence upon complexation; iii) assess the occurrence of FRET events between OTA and the Trp residue; iv) assess shifts in absorbance maximum of OTA, which are due to changes in the protonation state of the phenolic ring that are peculiar alteration of the micro-environment around the molecule. In order to investigate the nature of the potential interactions between peptide and OTA, all the measurements were performed varying the pH, changing thus the protonation state of the molecules involved. The peptide exhibits a quenching effect of the Trp residue in some experimental conditions: the best result was achieved at low temperature and basic pH.

Further characterization steps include: i) circular dichroism studies to evaluate the pre-existing peptide conformations and relative changes upon toxin complexation; iii) development of HPLC assays as a “non-spectroscopic” based method for complexation studies; iv) NMR analysis to acquire detailed structural information. Simultaneously, other reported sequences are under evaluation and a strategy for the peptide design is going to be set up. Improvements will also be investigated throughout fine-tuning design approaches that include insertion of modified aa, introduction of structural constrains and integration with other class of molecules. Despite some limitations, peptides should represent an valuable alternative to some Ab-based methods because they are cheaper, easier to produce, more chemically customizable and less prone to variability. Moreover, focusing the investigations on revealing the events that drive molecule interactions might represent a relevant driving force that push this topic further.

References
DETECTION – P26

Chlorophyll fluorescence and hyperspectral imaging detect fungal diseases on cereals within distinct time frames

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Fusarium infections on ears of cereal can cause yield losses. Mycotoxin contaminated products made from infected cereal grain can cause serious health problems in human and animal. At present, the mycotoxin content of grains are determined by time consuming and expensive laboratory methods like Fast-DON-ELISA-tests and HPLC at the harvest time. The early recognition of head blight infected ears together with GPS-based disease mapping could be helpful for a selective separated harvest of mycotoxin loaded crops.

The possibility of rapid non-invasive detection of Fusarium culmorum-diseased wheat ears was tested with two recent imaging techniques in the laboratory. Chlorophyll fluorescence image analysis (CFIA) enables the comprehensive evaluation of the photosynthetic activity of plants, which, in turn, is close related to their intactness. Statistical analyses of CF images applying the ‘cumulative Fv/Fm-value at 0.3’ could quite exactly establish the actual degree of disease of ears. With CFIA, the disease could already be verified after six days after inoculation at a symptoms threshold of 5%. In diseased plants, DON contents highly correlated with rated degree of disease and were beyond the limits licit in EU. In its current technical status, CFIA successfully detected head blight also under field conditions, though slightly less effectively.

As a second technique, a hyperspectral line scanner system working in the wavelength range of 400 nm to 1000 nm was evaluated. Time series of images of healthy and artificially Fusarium-infected ears were recorded and analysed with the image analysis software ENVI to define disease specific spectral signatures. Specific object classes were built as basis for classification of diseased and healthy ears. With ‘Spectral Angle Mapper’ as an appropriate classification tool, healthy and diseased ears could be clearly distinguished. However, this technique could not distinguish diseased and healthy ears if plants are in the growth stage BBCH 65 (end of blooming) and during ear maturation (BBCH 85). Nevertheless, within a time frame of about ten days (BBCH 71 to 77), a reasonably good disease detection is possible with both imaging techniques.

The fusion of both methods can largely improve the accuracy of Fusarium symptoms detection. This goal shall be achieved in future research project, hopefully with the help of highly committed interested companies.

References
DETECTION – P27

Analysis of mycotoxins in complex matrices by enzyme immunoassays

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The presence of mycotoxins in agricultural commodities is a matter of concern for both human and animal health. Rapid screening methods such as enzyme immunoassays are a valid tool for the screening of mycotoxins, thanks to their easiness to use, reliable results and low costs. However, some feed matrices could be particularly difficult to analyse because of their complexity due to the presence of high amount of proteins, fat, fibre, or because of their production process. The consequence is often low accuracy and precision of the results.

The performance of Tecna’s Celer ELISA kits was evaluated in the analysis of some complex matrices such as feed, cottonseed, corn germ and DDGS (Distilled Dried Grains with Solubles). Samples were homogenized by grinding, mixed with 70% methanol in the presence of sodium chloride, shaken for 3 minutes and filtered, in order to obtain a clear extract. Different mycotoxins were analysed in different matrices: T2 toxin in poultry feed; deoxynivalenol in pig feed and DDGS; aflatoxins in feed, corn germ and cottonseed; zearalenone in corn DDGS. Results of dosage of both spiked and naturally incurred samples were evaluated in terms of accuracy (recovery %) and precision, under repeatability as well as reproducibility conditions (intra-assay and inter-assay CV%). The sample preparation procedure turned out to be suitable in most of cases, obtaining mean recovery rates between 80 and 113%, and low CV values (intra-assay less than 12%, inter-assay less than 16%). In some cases, accuracy and precision have to be improved and in these cases modifications of the procedure was introduced. In the determination of aflatoxins in feed, cottonseed and corn germ, a clean-up of the methanolic extracts was necessary. For this purpose, Tecna’s EasyPur AFLA was successfully applied as a tool to lower matrix interferences. In the case of analysis of deoxynivalenol and zearalenone in DDGS, the sample extraction time was increased to obtain higher recovery rates.

Validation results indicate that Celer ELISA kits are rapid, accurate and precise screening methods for the analysis of mycotoxins also in complex matrices, according to performance criteria for methods of analysis of mycotoxins laid down by Commission Regulation No 401/2006.

References
DETECTION – P28

Automated highly sensitive method for Aflatoxin B/G or Ochratoxin A clean-up and analysis – a novel approach in mycotoxin analysis

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Mycotoxins are secondary metabolites from various fungi with highly toxic impact on humans and animals. The analysis of food and feed is regulated by law and consists of sampling, extraction, clean-up and analysis. The extraction of aflatoxins and ochratoxin A from matrices is achieved by organic solvents, the sample clean-up and concentration of the toxin by using immunoaffinity columns based on antibodies or SPE columns. The immunoaffinity clean-up is very common because of its specificity and the reduction of unspecific matrix components which could interfere with the subsequent HPLC fluorescence or LC/MS analysis. Toxins are eluted from the antibody by organic solvents, which denature the antibody and release the toxins. Eluates could be either evaporated and redissolved in HPLC solvent or are directly analysed by HPLC. For HPLC analysis only small volumes of organic solvents can be injected, or the eluate from the immunoaffinity column needs to be diluted or evaporated and redissolved in HPLC solvents, respectively. In any case large volume injection leads to chromatographic problems which could disturb the analytic result and the precision.

A new immunoaffinity column type for rapid clean-up was developed for aflatoxin and ochratoxin A analysis, which is combined with a new technique for elution of the toxins by elevated temperature denaturing the antibody with total release of the toxin. In combination with water based elution buffers a chromatographic focussing of high volume injections could be achieved on the HPLC which allow analysis of very low toxin amounts with good chromatographic separation and analysis. This new technique allows an ultimately fast clean-up compared to traditional immunoaffinity clean-up columns and a total injection of the eluate into the HPLC. This complex principle was fully automated and can be applied to matrices like nuts, peanuts, pistachios or cereal based matrices, and even spices. A sensitivity down to 10 parts per trillion (ppt) for the individual aflatoxins (B/G) can be achieved.

The similarly designed immunoaffinity column for ochratoxin A allows the clean-up of OTA from cereal based matrices, coffee, beer, wine, fruits, animal feed or spices with a detection level of less than 30 parts per trillion (ppt) by using regular fluorescence detection.

Thus it is an ideal tool for analysis of baby or dietary food matrices which are regulated at low levels for aflatoxin B/G or ochratoxin A within the EU. The increase in sensitivity provides a further reduction of the sample load with only 1 mL sample needed, representing less than 0.028 gram matrix equivalents with still excellent chromatographic results.

This approach was designed into an instrument called ThermELUTE enabling the analyst to achieve a sensitivity at ppt levels with fluorescence detection for all matrices regulated in a very short time.

Mycotoxin analysis with the ThermELUTE system is compatible with AflaCLEAN SMART or OtaCLEAN SMART columns.
The development of monoclonal antibodies against Deoxynivalenol

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Fusarium toxins called trichothecenes form the most commonly found mycotoxin group in Europe, among them deoxynivalenol (DON) occurs at highest rate. There is a need of a biosensor which can determine DON contamination on-site. For the biosensor, an immunoassay technology is chosen. Therefore, specific monoclonal antibody against DON with minimal cross reactivity against other mycotoxins like 3-ADON or 15-ADON will be developed.

An important aspect in the production of specific antibody, is the use of a good conjugate for immunization. The kind and size of linker, the location of the linker on the DON molecule will all have an influence on the kind of antibody that is obtained. Therefore different DON conjugates were made. As a first attempt, DON-hemiglutaryl (DON-HG) was synthesized. By using butylboronic acid as a chemical protection of C15, DON-3-HG was synthesized. To avoid cross reactivity of the antibody, other linkers without carboxyl function were used. A new linker for DON immunogen synthesis, was cyanyric chloride (CC). A protocol for DON-CC synthesis was made and the presence of DON-3-CC and DON-15-CC was determined using MS. To overcome the problem of cross reactivity, other functional groups out of the hydroxyl group on C3 and C15 of the DON structure were used for the synthesis of a hapten. Carboxy methyl oxime (CMO) and carboxy propyl imine (CPI) were coupled to DON via the ketofunction of DON. Compared to CMO, CPI is more natural and therefore has less chance to interact with the immune system of the mouse. So only DON will be the immunogenic part of the hapten.

All haptons were coupled to bovine serum albumin (BSA) and after determination of the conjugation ratio using ELISA, BCA and TNBS tests, they were injected into 6-week old Balb/C female mice. Hybridomas were formed out of the fusion of mouse spleen cells and NSO cells. These hybridomas were screened with indirect ELISA for the production of the target antibody using DON-ovalbumin (OVA), DON-HG-OVA, DON-CC-OVA and DON-CMO-OVA as coating antigens.

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DETECTION – P30

Quantification of mycotoxins in matrices with high sugar content by LC-MS/MS after liquid/liquid extraction

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Consumption of food and feedstuff contaminated with mycotoxins is recognized as a health risk. While major food components such as cereals and rice have been extensively analysed for the content of mycotoxins in the last decades, food products with a high sugar content have only recently become target of food quality monitoring (Delage et al., 2003; Malachova et al., 2012).

Analysis of samples with high sugar content such as sugar beet molasses, honey and fruit juice concentrates for mycotoxin content is complicated by a high viscosity of the samples. Such samples are difficult to pipet and block SPE columns during clean-up. Sugars may precipitate in extracts with a high content of organic solvents. When HPLC with mass spectrometric detection is used as an analytical method for these samples, electrospray ionization (ESI) poses a particular challenge because high temperatures inside the ionization chamber cause the sugar to caramelize, impacting the performance of the ion source and eventually blocking the needle of the electrospray.

As a solution to these problems, traditional liquid/liquid extraction was adopted. Liquid samples with high sugar content were diluted with sodium acetate buffer (pH 4) and extracted with ten volumes of ethyl acetate. The organic phase was dehydrated with molecular sieve and solvent war removed in vacuum. The residue was dissolved in mobile phase and analysed by HPLC-MS/MS.

Performance parameters of the method were determined for Fusarium mycotoxins produced in the field such as trichothecenes A and B, fumonisins and zearalenone, and for mycotoxins produced by storage fungi such as ochratoxin A and aflatoxins. Efficient removal of sugars was demonstrated, accompanied by reasonably good performance parameters of the method for target mycotoxins.

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References
DETECTION – P31

The use of ultra-performance convergence chromatography – tandem mass spectrometry (UPC²-MS/MS) in mycotoxin analysis

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The ultra-performance convergence chromatograph (UPC²) is a robust and reproducible supercritical fluid chromatograph (SFC) recently introduced by Waters (Milford, MA, USA) and able to work with sub 2 µm particles. A supercritical fluid is a substance at a temperature and pressure above its critical point, resulting in a substance which is neither a liquid nor a gas.

In practice supercritical CO₂ is most often used as mobile phase to separate the analytes of interest. As a supercritical fluid CO₂ has a low viscosity allowing the use of higher flow rates which results in faster analysis or the use of longer columns resulting in an increased resolution. By combining SFC with sub 2 µm particles the analysis time or resolution can even be further improved. Moreover, according to the manufacturer UPC² has a selectivity which is orthogonal to both reversed and normal phase HPLC, making this technique complementary to HPLC/UPLC. Furthermore, by using CO₂ as the liquid phase in chromatography the amount of used organic solvents can be reduced, making UPC² a green chromatographic technique.

Due to the very different physico-chemical properties of mycotoxins it has proven very challenging to determine different mycotoxins simultaneously. Although the most problems in the development of such a multi-mycotoxin method are situated in the extraction and clean-up steps, the use of HPLC/UPLC has also sometimes shown its limitations. For instance Monbaliu et al. (2009) showed that the separation of the isomers 3-acetyldeoxynivalenol (3-ADON) and 15-acetyldeoxynivalenol (15-ADON) (type-B trichothecenes produced by Fusarium species) using HPLC was impossible and that the differentiation between these two compounds can only be made based on the detected ion ratios. This however can be very difficult depending on the analysed matrix.

This poster will focus on the possibility and the added value of using UPC²-MS/MS for the first time in the analysis of mycotoxins.

References
DETECTION – P32

PCR-based detection of Aflatoxin-producing fungi in stored food products

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Aflatoxins are largely produced by Aspergillus flavus and A. parasiticus besides other Aspergillus species as well as other genus. Aflatoxin-producing fungi, in particular, Aspergillus flavus and A. parasiticus infect 40-50% of the crops such as maize, cotton, groundnuts, as well as tree nuts such as Brazil nuts, pistachio nuts, and walnuts1. In developing countries like India, where climatic and crop storage conditions are frequently conducive to fungal growth and mycotoxin production, much of their population relies on subsistence farming or on unregulated local markets. Thus, the food contamination remains an important issue in such countries. The development of sophisticated kits for the detection of minute amounts of aflatoxins is the most important step towards safer foods and feeds in these countries. Conventional detection methods (chromatographic and serological) are labor-intensive, time-taking, and requires days. Moreover, these models can only be used only after the toxin production. Due to the poor presentation of these conventional methods, scientists have shifted their attention to discover the direct detection methods for fungal pathogens2.

In our experiments, A. flavus MTCC 277, A. parasiticus MTCC 2796, A. flavus JH11, A. versicolor MTCC 280, Tricoderma viride MTCC 3114 and Fusarium oxysporum f. sp. ciceri were used for the study. Out of them, only A. flavus MTCC 277, A. parasiticus MTCC 2796 and A. flavus JH11 showed the production of aflatoxin B1 production. Consensus sequence of 236 bp from an exonic region of aflP gene was used for the amplification3. After optimization of in vitro amplification conditions, specificity was determined and only A. flavus MTCC 277, A. parasiticus MTCC 2796 and A. flavus JH11 showed the positive amplification with aflP/ aflP-R primers. The aflatoxin positive fungal DNA solutions were serially diluted for the assessment of limit of detection (LOD). As little as 100 femtogram (fg) of A. flavus MTCC 277 and 1 pg (pg per microlitre) of A. parasiticus MTCC 2796 and A. flavus JH11 diluted DNA was amplified by PCR. After this, amplification of serially diluted fungal spores (102, 104, 106, 108) revealed the detection of A. flavus, A. flavus JH11 and A. parasiticus with as low as 104 fungal spores whereas contaminated peanuts with different spore dilutions (102, 104, 106, 108) incubated at different time intervals (0, 12, 24, 36, 48 h) showed the threshold limit with 108 spores at 0 h. Conclusively, with this method, we can detect the aflatoxin producer species in contaminated agricultural products at the time of infection even in the presence of other fungal agents and the developed method is reliable, specific and much more sensitive than other conventional methods used for the detection.

References:
DETECTION – P33

Advances in analytical capability for the trace level detection of mycotoxin contamination in animal feedingstuffs

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There are now over 400 recognised mycotoxins found in foods and animal feedings materials and it has been reported that as much as 25% of the world’s cereal grains may be contaminated with mycotoxins. The majority of mycotoxins are extremely stable and not destroyed by food processing or cooking and as such can easily enter the marketplace. At present, mycotoxin contamination represents the highest number of notifications under the EU Rapid Alert System for Food and Feeds (RASFF).

The analysis of animal feeds including silage represents a major analytical challenge due to the complexity and in-homogeneity of these matrices. Although permitted limits for mycotoxins are set at relatively high (mg kg⁻¹) concentrations in the EU, toxic effects, e.g. immunotoxicity and feed uptake problems in certain species (poultry and porcines) are often observed at low (µg kg⁻¹) concentrations. For this reason there is often an economic requirement to achieve low detection limits in this matrix. There is also a high potential for co-contamination in this matrix (due to pre and post harvest infestation) resulting in the occurrence of tricothecenes, aflatoxins, fumonisins, ochratoxin, patulin, T-2/HT-2 and alternaria toxins for example within a single sample.

Within this paper we report the development of a quantitative method for the determination of circa 35 relevant mycotoxins in a variety of animal feed and silage extracts. A generic and simplified sample extraction protocol based on 84:16 (v/v) acetonitrile:acidified water for the recovery of mycotoxins was used, and analysed using an ACQUITY UPLC and Xevo TQ-S tandem quadrupole mass spectrometer. We investigate the affect of matrix dilution coupled to high instrument sensitivity to overcome common analytical challenges such as ion suppression. We also exploit the functionality of the Xevo TQ-S: to monitor background interference profile simultaneously during MRM transitions; the use of product ion confirmation to provide additional confidence in identifications; and Quanpedia for automated MRM scheduling and method development.
DETECTION – P34

Synthesis of multicolored labels based on CdSe quantum dots for immunochemical mycotoxin detection

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Semiconductor quantum dots (QDs) are became popular as fluorescent biolabels in immunochemistry due to their unique optical properties. Opposite to known organic fluorescent dyes, QDs have a symmetric and narrow band which enables emission of pure color. In addition, their absorption spectrum reaches into the UV regardless of their size. So, their spectral characteristics allow simultaneous excitation of different particle sizes at a single wavelength with emission at multiple wavelengths. We chose CdSe as a main component of QDs because nanoparticles of CdSe’s crystals emit light in a visible region of the spectrum. Hydrophobic QDs (CdSe/ZnS (λ(em)=520, 540 nm) and CdSe/CdS/ZnS (λ(em)=570, 590, 610, 640 nm)) were prepared by high-temperature synthesis in organic solvent octadecene. The quantum yields of obtained QDs were 52% (CdSe/ZnS) and ~40% (CdSe/CdS/ZnS) and the full width at half maximum of their fluorescence peak doesn’t exceed 30 nm.

QDs used in biomedical research have to be water-soluble because QDs conjugation with biomolecules occurs in water solutions. Method of QDs coating by amphiphilic polymers was developed and worked out for QDs with green, yellow, orange and red emission’s colors. This method is good because hydrophobic ligands aren’t removed from QDs surface during solubilization process so the QD brightness of fluorescence doesn’t decrease dramatically after transformation to water solution.

In the case of mycotoxin’s detection it’s important to define several toxicants simultaneously in one sample. Obtained QDs with different emission colors can be used in quantitative micro-well plate immunoassays (fig.1A) and qualitative column test (fig.1B) for simultaneous mycotoxin’s detection.

Fig.1. Scheme for developing methods for multi-mycotoxin detection on separate test zones: (A) - microtitre plate FLISA and (B) column-based rapid field test; 1 – application of standard solution or sample and 2 – application of conjugates labelled with QDs of different colour after washing step

Acknowledgement
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DETECTION – P35

Simultaneous determination of deoxynivalenol, zearalenone and their metabolites in bovine urine

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Zearalenone (ZEN) and deoxynivalenol (DON) are mycotoxins, which are formed by various Fusarium species on cereals. They might have negative effects on animal health and performance when toxicologically relevant diet concentrations are exceeded. These mycotoxins and their degradation products can potentially be used as biomarkers in urine to assess the exposure of humans and animals. However, faster, cheaper and more practical multi-toxin methods with lower detection limits are needed. Therefore, the aim of this study was to develop a method for ZEN, DON and their metabolites alpha-zearalenol, beta-zearalenol, zearalanone, alpha-zearalanol, beta-zearalanol and de-epoxy-DON in bovine urine, which combines an economic sample preparation and the selectivity and sensitivity of LC-MS/MS.

A variety of methods for the preparation of urine has been investigated, including solid-phase extraction (SPE), dilute and shoot and QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe)-based method. By comparing the recoveries the most suitable method was worked out. It consists of an extraction by SPE using Oasis HLB cartridges (Waters) and methanol as elution solvent. Different washing solutions were tested to optimize the SPE technique. Furthermore, different dilutions of urine were analysed to find a compromise between toxin concentration and matrix effects. All samples were treated by an enzymatic hydrolysis with beta-glucuronidase (Type H-2, Sigma).

Sample analysis was performed on an Agilent 1200 series HPLC system using a Pursuit™ XRs Ultra 2.8 (Varian) column coupled with a 4000 QTrap (Applied Biosystems) LC-MS/MS system with negative electrospray ionization (ESI). The developed method showed recovery rates ranging from 30-70%. By using of 13C-labelled and deuterated internal standards (alpha-ZEL-d₆, beta-ZEL-d₆, alpha-ZAL-d₆, beta-ZAL-d₆, ¹³C₁₈-ZEN und ¹³C₁₈-DON) the recovery was corrected up to 70-110%.

The usability of the developed method was demonstrated by the analysis of spiked samples of bovine urine. Analyses of urine samples obtained from different feeding trials and the validation of this method are in progress.
DETECTION – P36

A comparison of the ELISA and the HPLC analysis of ochratoxin A in human blood serum in the Czech Republic

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Introduction:
Ochratoxin A (OTA) is an important mycotoxin produced by microfungi of the species \textit{Aspergillus} and \textit{Penicillium}. Apart from its marked nephrotoxicity, OTA displays hepatotoxic, teratogenic, carcinogenic and immunosuppressive properties. The risk to human health results especially from the intake of OTA through the contaminated foods of both vegetable and animal origin. OTA has been detected i. a. in human blood and mother’s milk.

Methods for data collection and analysis
In total, 115 blood samples of the Czech population were collected. The age of volunteers ranged from 19 – 40 years with the mean age of 31 years. All samples were collected during 2012. Competitive enzyme immunoassay (Ridascreen Ochratoxin A 30/15 /Art. No.: R1311/, R-Biopharm AG, Germany) for the quantitative analysis of OTA in human blood serum was performed. The test kit is sufficient for 96 determinations including OTA standards. The measurement of OTA was made photometrically at 450 nm. Validated and accredited ultra-trace HPLC-FD method was employed for OTA determinations for possible comparison. The human blood serum samples were cleaned by means of immunoaffinity chromatography (OCHRAREP® columns, R-Biopharm, Germany). The detection limits were at 0.04 ng/mL for HPLC and 0.05 ng/mL (50 ppt) for ELISA.

Results
The results of the HPLC-FLD and ELISA methods were compared and the main statistical parameters included. Both data sets were comparable. Based on F-test evaluation, there seems to be no statistically significant difference between both of them ($F = 0.72 < 1.36$ /critical value; $p = 0.05$). The OTA was present in a broad range of concentrations from 0.037 to 1.130 ng/mL. The results of the ELISA and HPLC analysis correlated well enough (linear correlation: $y = 0.9379x + 0.0179$; $r = 0.907$ /$p = 0.05, n = 115$).

Conclusions
In this work, the commercial ELISA test kit was evaluated for purposes of determining OTA in human blood serum samples. The results in 115 samples were compared with the validated and accredited HPLC-FLD method. Based on the statistical results, it can be concluded that both the ELISA and HPLC methods are in a very good correlation ($r = 0.907$). The OTA was present in broad range of concentrations from 0.037 to 1.130 ng/mL.

Acknowledgement
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DETECTION – P37

Determination of Mycotoxins with RP HPLC in combination with Myco6in1™ immunoaffinity columns

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Today over 400 mycotoxins are identified, with deoxynivalenol, zearalenon, ochratoxin A, fumonisins and aflatoxins being the most investigated. Most mycotoxins are produced by Fusarium, Penicillium, Alternaria and Aspergillus species. This study was designed to develop a method for the simultaneous detection and quantification of 11 mycotoxins in feed and feed raw materials (or 6 types of mycotoxins): fumonisins: B1 and B2; aflatoxins: B1, B2, G1 and G2; ochratoxin A; zearalenon; trichothecenes: deoxynivalenol, T2 and HT-2. All 11 mycotoxins are bound to one immunoaffinity column (IAC). Immunoaffinity columns are used for their high selectivity of mycotoxin binding, which allows for a very efficient clean-up in complex matrices.

Immunoaffinity column Myco6in1™ from Vicam is compared to the standard IAC which is designed to bind one mycotoxin or one type of mycotoxins on one IAC. The Myco6in1 IAC has the advantage that only 1 extraction of each sample is required instead of different sample extractions for each type of mycotoxin. The search for a suitable solvent system was essential to achieve excellent recoveries. The extraction, purification with Myco6in1 IAC and HPLC analysis (HPLC-FL and HPLC-UV) result in consistent recoveries of mycotoxins in different matrices.

The limit of quantification (LOQ) is the same as those with standard IA columns. This method has been evaluated via proficiency tests and reference material. The use of the multimycotoxin immunoaffinity column yields to a 30% reduction of the analysis time.

References
Effect of alkaline-cooking process on the content of fumonisins and relevant hydrolysis products in maize

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Fumonisins B1 (FB1) and B2 (FB2) are toxic compounds produced mainly by *Fusarium verticillioides* and *F. proliferatum*, which frequently contaminate maize and maize products worldwide. Nixtamalization (alkaline-cooking) is an ancient art of cooking maize in a calcium hydroxide solution (lime) to produce a soft dough called *masa* that is used to make tortillas, tamales, tacos, burritos and snacks.

The effect of nixtamalization on the content of fumonisins and relevant hydrolysed and partially hydrolysed forms (HFB1, HFB2, PHFB1 and PHFB2) was investigated by reproducing the processing at laboratory scale. A mass balance calculation was applied to quantitatively estimate the fate of fumonisins during the processing. Nixtamalization experiments were carried out by cooking maize kernels at 90°C in presence of lime. Two levels of lime (1% or 5%) and different cooking times (0, 15, 30, 60 min) were used. Starting maize, intermediate fractions (steeping water and washing water) and final fraction (masa) were collected, freeze-dried for moisture content, ground and analysed by LC-HRMS for toxins content.

Fumonisins (FB1+FB2) and their partially hydrolysed forms (PHFB1+PHFB2) in starting maize materials ranged from 4 to 20 µg/g and from 0.10 to 0.50 µg/g, respectively, whereas no hydrolysed forms were found. Nixtamalization decreased fumonisins and partially hydrolysed fumonisins in masa by 32-66% and by 94-98%, respectively, depending on cooking time and lime levels. The highest reduction was observed when maize was cooked with 5% lime for 30 or 60 min. Moreover, during nixtamalization fumonisins and partially hydrolysed fumonisins were further hydrolysed as indicated by the presence of HFB1, HFB2 in the masa at levels from 0.06 to 0.75 µg/g. The mass balance calculation indicated that the total amount of the three forms of fumonisins collected in the intermediate fractions and in the masa accounted for 130-186%, of which 11-62% in the steeping water, 20-69% in the washing waters and 56-79% in masa. These findings clearly indicate that nixtamalization can make available forms of fumonisins that are conjugated or caged with matrix components, and can be converted to both hydrolysed and partially hydrolysed forms.

Acknowledgement
This research was supported by EU-FPVII project MYCORED (KBBE-2007-222690)
Dried fruits are highly susceptible to mould growth and consequently to mycotoxin contamination as it has been stated recently in some studies(1). The presence of mycotoxins in food may affect human and animal health as they may cause many different adverse effects such as induction of cancer and mutagenicity, as well as estrogenic, gastrointestinal and kidney disorders. The most commonly sample treatment used for the determination of mycotoxins in dried fruit involves the use of solid-liquid extraction followed by a clean-up step using immunoaffinity columns (IAC) but their inherent selectivity limits the multiclass analysis of these contaminants.

In recent years there has been an increasing interest in the development of methods supporting the so-called green chemistry, being simpler, more efficient and environmentally friendly in terms of reduction of organic solvents, contaminant waste and cost. Moreover, multiresidue methods able to monitor a high number of compounds in a single run according to the established legislation for many different matrices, are also very attractive.

In this work, a sensitive, simple and rapid method for the determination of fifteen mycotoxins in dried fruits (including almonds, peanuts, sunflower seeds, pumpkin seeds, nuts, macadamia nuts, pistachios, hazelnuts and pine nuts) based on ultra high performance liquid chromatography coupled with tandem mass spectrometry (UHPLC-MS/MS) has been developed and validated. The method covers all the mycotoxins included in the regulation (EC) 1881/2006 (2), and others considered as hazardous substances by the International Agency for Research on Cancer (3). The sample treatment comprises a first step based on QuEChERS procedure for the determination of fumonisin B₁, fumonisin B₂, nivalenol, deoxynivalenol, fusarenon-X, T-2 and HT-2 toxin, citrinin, stigmatocystin, zearalenone and ochratoxin A. A subsequent clean-up step based on dispersive liquid-liquid microextraction (DLLME) was necessary for the determination of aflatoxins (B₁, B₂, G₁ and G₂), since their determination was not possible applying only the QuEChERS based extraction. The method was fully-validated in peanuts as representative matrix of dried fruits, and was subsequently applied in the other eight dried fruit matrices. Quantification limits obtained for aflatoxins, the unique mycotoxins legislated on these matrices, were lower than the maximum levels allowed by the current legislation, while quantification limits obtained for the other mycotoxins were lower than the limits usually permitted by the legislation in other food matrices. Precision of the method, expressed as RSD (%), was always lower than 11%, and recoveries ranged between 60.7% and 104.3%, except for nivalenol for which a low recovery was obtained.

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Natalia Arroyo-Manzanares thanks the “Junta de Andalucía” for a predoctoral grant. JFHP thanks the Ministry of Economy and Competitiveness of the Spanish Government for a Juan de la Cierva postdoctoral contract.

References
Studies on PR-toxin from *Penicillium roqueforti* cultures for the preparation of an analytical reference standard

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PR-toxin is a toxic metabolite from *Penicillium roqueforti*, a greenish-blue sporulating fungal species used in ripening of blue cheese (eg. French Roquefort cheese, Italian Gorgonzola, English Stilton, Spanish Cabrales or German Bavaria Blu). It is known, that *P. roqueforti* produces several metabolites like roquefortine C, mycophenolic acid and PR-toxin which are more or less toxic. According to the literature, from all its metabolites, the highest toxicity seems to have PR-toxin.

Quality controls of foodstuffs are indispensable and particularly in the case of the production of blue cheese, it is necessary to monitor its ripening to control for potential appearance of toxic substances like PR-toxin. Furthermore PR-toxin can also be found in molded hay and maize silage and spoiled grains. Contaminated hay or poorly ensiled crops can cause fatal poisoning in livestocks and require testing for mycotoxins contamination.

Due to the lack of a commercially available reference standard for PR-toxin, a project was initiated to isolate and purify PR-toxin from a selected culture of *P. roqueforti* for the preparation of analytical reference standards. During the isolation of PR-toxin, several metabolic precursors and degradation products were found. Also the formation of an isomer – depending on the solvent – was observed. The sensitivity against UV-Light, protic solvents and the instability of the toxin in the presence of amino compounds complicated the isolation of the pure substance. The estimated purity of the final purified PR-toxin, by using NMR and the chromatograms of HPLC-UV and MS, was higher than 95%. To admit the substance as reference standard, further stability studies have to succeed.
DETECTION – P40

Studies on PR-toxin from Penicillium roqueforti cultures for the preparation of an analytical reference standard

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Lateral flow devices for the semi-quantitative detection of the mycotoxin Zearalenone in corn by using “green extraction”

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Zearalenone (ZON) belongs to the most predominant Fusarium toxins found in food and feed. Besides standard analytical methods, screening methods are increasingly needed. Immunooassay-based methods, for example lateral flow devices (LFDs), also known as strip tests, allow qualitative or semi-quantitative determination of mycotoxins within a few minutes. Because of the ubiquitarry occurrence of mycotoxins in cereals and their various toxic effects on human and animals, authorities such as the European Commission or the Grain Inspection, Packers and Stockyards Administration (GIPSA) have addressed the mycotoxin problem by adopting regulatory limits.

Sample preparation for testing mycotoxins in food and feed includes extraction of the analytes from the commodities (e.g. corn). This is mostly performed with organic solvents like methanol or acetonitrile, due to insolubility of most mycotoxins in polar solvents. The use of rapid strip tests for on-site screening is getting more and more common. When samples are extracted on-site, people have to work with organic solvents with little protection and as such they might be exposed to these substances. Organic solvents are toxic and harmful to human health and the environment, particularly when large amounts of solvent are used for sample extraction. Therefore the reduction of solvent consumption or complete replacement of organic solvents is an important and relevant issue in rapid test performance.

The implementation of an extraction procedure using less toxic, i.e. “greener” solvents for use in LFD systems for the detection of Zearalenone in corn turned out to be a complex challenge and resulted in a novel extraction method that employs less toxic solvents and newly developed, aqueous buffer systems.
DETECTION – P42

Analysis of total ergot alkaloid amount in various crop samples by ELISA

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Ergot alkaloids are secondary metabolites of various fungal species belonging to the genus Claviceps, which grow mainly on wheat, rye, triticale and other grain species. Ergot alkaloids are known to be highly toxic for humans and animals and contaminated cereals could cause severe intoxication and neurotoxic symptoms. This is the reason for better control and screening tools for these crops. Today concentrations of ergot alkaloids are assumed from the amount of sclerotia in the crop, were in the EU regulation 824/2000 a maximum of 500 mg Sclerotia per 1 kg (0.05 % w/w) is tolerated. In assumption on the mean total ergot alkaloid concentration of 0.2 % in sclerotia, the maximum tolerated level of total ergot alkaloid would be 1,000 microgram per kilogram.

In 2005 the European Food Safety Authority (EFSA) commented the discrepancy in acceptable and practically useable procedures and the need to measure ergot alkaloid concentrations. Until now, various time consuming methods have been established mainly based on LC/MS analysis. An alternative method to this time consuming methods is the analysis of ergot alkaloids by Enzyme-linked Immunosorbent Assay (ELISA).

A new indirect competitive ELISA for estimation of the total alkaloid concentration, including the different -ine and –inine epimers, in various crop materials with a cut-off of 1,000 µg/kg is presented. As an example data from wheat, rye and triticale samples correlated with LC/MS are presented, which show a very good correlation and indicate the suitability of the ELISA as screening tool for ergot alkaloid analysis.

In comparison to LC/MS analysis and sample cleanup the ELISA needs less than two hours and allows parallel analysis of several samples, the ELISA could be performed by trained person, who are familiar with ELISA techniques and does not need expensive analytical lab equipment.

For evaluation purposes real contaminated wheat, rye and triticale samples were tested.
DETECTION – P42

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DETECTION – P43

Simultaneous determination of total Aflatoxin, Ochratoxin A and Zearalenone using AO ZON PREP® in conjunction with HPLC

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European legislative limits for total aflatoxin, ochratoxin A and zearalenone are in place for cereals and cereal products. Aflatoxin in animal feed is also legislated for with guidance levels available for ochratoxin and zearalenone. There is a demand in the market for faster and less labour intensive tests. Immunoaffinity columns are rapidly becoming the routine standard method of choice for complying with regulatory mycotoxin analysis however there is a growing need for multi-mycotoxin analysis using a single extraction method.

In response, R-Biopharm Rhone has produced a multi-toxin immunoaffinity column, AO ZON PREP® enabling the isolation and concentration of aflatoxins B1, B2, G1, G2, ochratoxin A and zearalenone in difficult commodities such as animal feed. Two HPLC conditions were developed; one for the simultaneous detection of total aflatoxin and ochratoxin A based on the use of the KOBRA® CELL for derivatisation and a second for zearalenone. The advantage of this new immunoaffinity column is that only one sample preparation method is required for all six mycotoxins therefore having greater sample throughput and a reduction in the use of solvents and consumables.

The method was validated in-house using AO ZON PREP® to test various animal feed samples. Extraction was with 60% acetonitrile, samples were then diluted with PBS before being passed through the immunoaffinity column where they were washed and finally eluted with methanol before being injected onto the HPLC system. Chromatograms of all samples processed using the AO ZON PREP® columns contained less interfering peaks and the method was found to surpass the current EU legislative limits and was shown to perform in line with current EU performance criteria. AO ZON PREP® can provide a rapid and robust method of analysis enabling accurate, low level quantification of all six mycotoxins in various animal feed samples.
Mycotoxin calibrants - an underestimated source of systematic errors?

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Reliable figures from mycotoxin determination are a key element to make sound decisions for all scientists. This is the reason why method performance characteristics have been established by various organisations such as ISO, CEN, AOAC and European legislation and methods have to prove to fulfil these provisions. It is thought that the main contributor to the dispersion of results between laboratories (Reproducibility or RSDR) is linked to parameters associated to the implementation of the method in various laboratories. The Joint Research Centre (JRC) in its function as European Union Reference Laboratory (EURL) for mycotoxins conducted a study with National Reference Laboratories (NRLs) that allowed the conclusion that the preparation of calibrants led to a much higher dispersion of results than that of the analytical method itself, including extraction, clean-up and chromatographic determination.

As a result the EURL has conducted further proficiency tests for different mycotoxins and gathered data that allowed a deeper investigation which role the quality of calibrants plays in the dispersion of results between laboratories. The poster highlights the effects observed, the parameters that are thought to be relevant for calibrant preparation and which methods laboratories can use to verify the quality of their calibrants.
DETECTION – P 44
Mycotoxin calibrants - an underestimated source of systematic errors?

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Commitment is our main ingredient.
Precise, Numerical Results

Countries around the world are continuing to introduce new legislation requiring control of mycotoxin levels in food and feed. At the same time, research is confirming that different mycotoxins in the same product can act synergistically to cause more harm than would be expected from simply adding the effects of the individual toxins. As a result, laboratories must be equipped to detect and quantify multiple toxins simultaneously in food and agricultural products. VICAM, the world’s leading provider of mycotoxin testing solutions, has developed a first-of-its-kind multi-mycotoxin immunoaffinity (IA) column for the simultaneous detection of aflatoxins, ochratoxin A, fumonisins, deoxynivalenol, zearalenone, T-2, HT-2, and nivalenol. Designed for use with LC/MS/MS detection — one of the most advanced, accurate, and widely used analytical methods in the world — VICAM’s Myco6in1™ LC/MS/MS IA column enables laboratories to simultaneously determine multiple mycotoxins with unmatched sensitivity, selectivity, and speed.

The patented* Myco6in1™ LC/MS/MS IA column is a quantitative tool which combines the proven strengths of selective monoclonal antibodies and liquid chromatography combined with tandem mass spectrometry to deliver precise numerical results. Simple, streamlined, and easy-to-use, the Myco6in1™ LC/MS/MS IA column requires only one sample and a single pass through the column, minimizing time and materials costs while improving laboratory productivity. VICAM continues to provide the optimized technology that delivers high recoveries, accurate results, and the reliability you’ve come to expect.

BENEFITS

- **Exclusive** – For use with LC/MS/MS and HPLC with PDA, fluorescence detector and post-column derivatization
- **Accurate** – Meets European Committee for Standardization (CEN) criteria for mycotoxin analysis methods
- **Comprehensive** – Detects aflatoxins, deoxynivalenol, fumonisins, ochratoxin A, zearalenone, and T-2 and HT-2 toxins with one simple test
- **Convenient** – One sample, one pass through the column to detect multiple mycotoxins
- **Durable** – Long shelf life
- **Economical** – One test provides results for multiple toxins, saving time and materials

**SAMPLE EXTRACTION**
- Grind and weigh sample
- Extract
- Centrifuge
- Evaporate extract

**CAPTURE AND ELUTE**
- Re-dissolve in PBS
- Pass extract over column
- Wash column
- Elute toxins and collect in cuvette

**QUANTITATE**
- Dry down and reconstitute eluate
- Inject onto LC/MS/MS
Multiresidue mycotoxin analysis of conventional and organic agriculture commodities by UPLC-MS/MS

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In 2011 the organic farming, which is continuously growing, reached almost 12% of the farmland acreage in the Czech republic. The crops of great importance are cereals followed by fodder crops. The organic wine market is increasing and it is becoming popular as well. Organic products have been grown in compliance with principles of organic farming that typically excludes the use of chemicals as fertilizers, fungicides, herbicides and other types of pesticides. The study deals with analysis of mycotoxins in organic and conventional farm products such as raw materials for feed production and grapes. The multiresidue mycotoxin method for feed analysis by ultra performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS) which has been recently developed represents useful tool for both feed and fruit analysis with certain modifications during the sample preparation. The method for determination of 17 mycotoxins (deoxynivalenol, nivalenol, HT-2 toxin, T-2 toxin, ochratoxin A, zearalenone, aflatoxins, fumonisins, beauvericin and enniatins) based on unbuffered QuEChERS method has been validated. The study presents results of mycotoxin screening performed on grape samples of conventional and organic origin produced in 2011 and 2012 and raw feed material samples of conventional and organic origin produced in 2012.
PRODUCTION AND OCCURRENCE – P46

Total Synthesis of AOH- and AME-beta-D-Glucosides

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Herein we present the total synthesis of several alternariol (AOH) and alternariol-9-methyl ether (AME) beta-D-glucosides (Glc) originated from 3, 5-dimethoxy bromobenzene (1) and orcinol (2). Via this synthetic route the constitutional isomers of the desired conjugates AOH-3-Glc and AOH-9-Glc as well as AME-3-Glc are accessible in a way that an intermingling is impossible. Samples of these conjugates already serve as reference materials.

Figure 3 Synthesis of the alternariol scaffold yielding ADME and ADBE

The build-up of the AOH scaffold by our approach leads to the 7, 9-dimethyl ether (ADME) or the 7, 9-dibenzyl ether (ADBE) of alternariol, depending on the protocols used during total synthesis (Fig. 1). Starting from these compounds all mentioned and also further conjugates (e.g. sulfates) are accessible (Fig. 2). The use of a triisopropylsilyl group (ASE & ADMSE) allows selective glucosylation at position 9 and their convenient cleavage by fluoride ions spares the glycosidic bond. Also the benzyl groups of ADBE can be cleaved orthogonally to that joint. Final saponification under optimised conditions afforded the desired alternariol glucosides.

Figure 4 Synthesis of the desired glucosides (Bn = benzyl, TIPS = triisopropylsilyl, Me = methyl)

Reagents and conditions: a) Acetobromoglucose, AgO, ACN; b) Pd/C, H2, MeOH/DCM; c) TBAF, AcOH, THF; d) TIPS-Cl, imidazole, DMF; e) AlCl3, NaI, ACN; f) BBr3; g) KOH, THF/H2O;
PRODUCTION AND OCCURRENCE – P47

Contamination of Zearalenone in animal feeds distributed in South Korea

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Introduction Zearalenone (ZEN) is one of the most widely distributed mycotoxins produced by Fusarium species (F. graminearum, and F. culmorum). ZEN is found worldwide in various cereals such as maize, barley, wheat and sorghum. ZEN is classified as an estrogenic mycotoxin because it frequently causes hyperestrogenic syndrome in animals. When ZEN-contaminated feed or grain was eaten by livestock, it can contribute to a wide variety of reproductive problems. Therefore, the objective of this survey was to monitor the occurrence and contamination level of ZEN in animal feed in order to increase food and feed safety.

Method The occurrence of ZEN was surveyed in 150 samples of compound feeds and 30 samples of single ingredient feeds that were supplied in South Korea. Immunoaffinity chromatography for purification and high-performance liquid chromatography (HPLC) were applied for ZEN detection and quantification. Feed samples were extracted with acetonitrile-water (75:25, volume per volume) and the extract was diluted with water (20:80, volume per volume) and applied to a Vicam ZearalaTest immunoaffinity column. All feed samples were analyzed using HPLC with fluorometric detection (Excitation=275 nanometer, Emission=450 nanometer) and methanol-water-acetonitrile (55:35:10, volume per volume) as mobile phase.

Result The contamination rate of total feed ingredients by ZEN was 89.4 percent. The highest contamination rate of ZEN (100 percent) was detected in compound feeds of cattle. The contamination rate of compound feed of poultry, swine and single ingredient feeds were 90, 94 and 63 percent, respectively. The average contamination level of positive compound feed of cattle, poultry, swine and single ingredient feeds were 134, 31, 24 and 56 nanogram per gram, respectively. The highest contamination level of ZEN (481 nanogram per gram) was found in fatting calves feed of feed samples.

Summary ZEN is a non-steroidal oestrogenic mycotoxin produced by F. graminearum that induces hyperoestrogenic responses in mammals such as swine. In this study, Contamination by ZEN was monitored in 150 compound feeds and 30 ingredient feeds that were distributed in South Korea. 161 samples were contaminated with ZEN out of 180 samples. This result indicates that most animal feeds distributed in South Korea are contaminated with ZEN. Therefore, continuous monitoring of ZEN will be necessary in feeds for food safety.

References

PRODUCTION AND OCCURRENCE – P48

Synthesis of Zearalenone-14-beta-diglucoside

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The formation of zearalenone-14-beta-diglucoside during metabolism of zearalenone (ZEN) in plants was reported by Berthiller in 2006. For further investigations in terms of bioanalytics and toxicology, reference material of this compound is still needed. Therefore we have synthesized ZEN-14-beta-diglucoside applying two different strategies. Preparation of the title compound via a direct approach required bromogentiobiose heptaacetate as donor for Koenigs Knorr glycosylation. This diglucosyl donor was synthesized from gentiobiose octaacetate, a beta-1,6-linked disaccharide, in a reaction with acetylbromide in methanol.

Additionally an appropriate acetimidoyl donor, used for Lewis acid mediated glycosylation of ZEN, was prepared by deprotection of the anomeric center of gentiobiose octaacetate with benzylamine, followed by a reaction with N-phenyltrifluoroacetimidoyl chloride (Fig. 1).

Pre-tests for glycosylation using a ZEN mimic have shown that Koenigs-Knorr reaction activated by silver(I)oxide in acetonitrile yielded the best results. Therefore this optimized method was applied for the first synthesis of ZEN-14-beta-diglucoside (Fig. 2) yielding the desired product in moderate overall yields.

Regarding the divergent approach, a 6-silyl protected glucosyl donor was prepared. Subsequent conjugation to ZEN led to a versatile intermediate that can be used for the synthesis of several metabolites. This is possible because the selective deprotection at position 6 allows further conjugation with any glycosyl donor desired (Fig. 3).

Mycotoxicological evaluation of barley and its products

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Barley (Hordeum L.), aside corn and wheat is one of the most commonly cultivated plants, making up 5% of the world and 20% of European cereal production. The increased interest in this plant can be observed mainly due to the development of beer production and ethanol fuel technologies. Yet, barley also constitutes nutritious feedstuff for animals and food commodities for humans (such as flour, flakes and grits). Wide application of this cereal brings about the need to protect it against moulds contamination and carry out regular mycotoxins content tests which, in favourable conditions, can be produced by fungi present not only in grain but also in final products.

The aim of the study was the evaluation of trichothecenes and zearalenone content in barley grain and food commodities such as grits, flour and flakes, coming from organic and conventional farms. The analysed material included 53 samples, with 31 from organic (15 - raw material, 16 - products) and 22 conventional farms (10 - raw material, 12 - products), respectively. The food products had been purchased in local shops in sujewsko-pomorskie province. Raw materials also came from the same region. Each sample was analysed in triplicate. Eight mycotoxins were determined using HPLC-MS/MS method: nivalenol (NIV), deoxynivalenol (DON), 3-acetyldeoxynivalenol (3ADON), monoacetoxyseirpenol (MAS), diacetoxyseirpenol (DAS), T-2 and HT-2 toxins and zearalenone (ZEN). Sample preparation was performed using Bond Elut® Mycotoxin columns (Agilent) and internal standards (IS) 13C-DON, 13C-T-2, 13C-HT-2, ZAN.

HT-2 toxin and NIV dominated in the analysed grains and food products (30 % and 28 % respectively), while 3ADON was not detected in a single sample. Among the 8 analysed mycotoxins it was NIV that dominated and its highest concentration was 85.6 ppb in the raw material from an organic farm. The highest number of mycotoxins was found in the grain from a conventional farm, with HT-2 and T-2 toxins prevailing (80 % and 70 % samples, respectively). Their concentration was also the highest in this group (13.8 ppb and 23.8 ppb, respectively). The third highest concentration turned out to be of DON (65.1 ppb). The highest concentrations of MAS and DAS were detected in raw materials (6.20 ppb in samples from an organic farm and < 3 ppb, in both groups, respectively). ZEN was most commonly present in food products coming from conventional farms - 42 % samples. The most contaminated product turned out to be barley flour, containing four out of eight analysed mycotoxins. The contamination of organic and conventional products was comparable. Mean concentration of the analysed mycotoxins was much lower in food products than in raw material (e.g. mean concentration of NIV was 2.5 ppb in organic products and 16.1 ppb in organic raw material). None of the analysed products contained 3ADON or DAS. Moreover, neither DON was detected in any of the organic products nor MAS in the conventional ones. No tested sample exceeded the maximum acceptable levels of mycotoxins (Commission Regulation 1881/2006 EC).

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Mycotoxins contamination of food in Thailand (2000-2010): Food safety concerns for the world food exporter

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Mycotoxins contamination of food has been a worldwide problem. These secondary metabolites produced by fungi contaminate various foodstuffs, from raw agricultural commodities to processed food. As the world food exporter, Thailand has to be aware of food safety. This study aims to evaluate the occurrence data on mycotoxins in food in Thailand. Both international and local publications from the year 2000 to 2010 were retrieved by using the following keywords: food, mycotoxin, aflatoxins, trichothecenes, deoxynivalenol, fumonisins, zearalenone, ochratoxins and patulin. A variety of food such as cereal grains, spices, animal products and ready to eat food have been reported. Among 28 studies, 22 studies (79%) included aflatoxins analysis. Ochratoxins, fumonisins and deoxynivalenol were reported in 5 studies (18%). T2-toxin and zearalenone were reported in 4 (14%) and 3 (11%) studies, respectively. Occurrence of patulin in food especially fruit and apple juice is needed in Thailand as there was no report. Aflatoxins have occupied the major area of mycotoxins investigation in Thailand. Aflatoxin contamination in peanuts has long been a major problem. Seventeen percent of 713 peanut product samples were contaminated with aflatoxins (0.7-3238 ppb). The other mycotoxins contaminated food in a relatively low level except ochratoxin. Eighty nine percent of dried Arabica coffee bean and 100% of dried Robusta coffee bean planted in Thailand were contaminated with ochratoxin. The highest level was 27µg/kg. Coffee is one of the major human source of exposure to ochratoxin besides cereals, wine, grape juice and pork. Interestingly, highly susceptible product like maize was not adequately studied. Some reports did not show either limit of detection or limit of quantitation. A key question about those reports is the reliability of results. In Thailand, regulatory limit has been set only for aflatoxins (AFB1, B2, G1, G2 at 20 ppb). As the food exporter, the agricultural commodities have to meet the regulation of the importing countries. However, with the strict action of those countries may lead to the difficulties of exporter to maintain the quality especially the year of extreme weather, drought and flood. It is possible that a low quality product will be left for local consumption instead of destroying or diverting. Local people are supposed to consume safe food as well even though the regulation in the country has not covered all of the major mycotoxins.

References
PRODUCTION AND OCCURRENCE – P51

Influence of climatic factors to the occurrence of *Fusarium* species and mycotoxins in wheat in Schleswig-Holstein

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Wheat is frequently attacked by *Fusarium* species causing *Fusarium* head blight (FHB). Flowering is known as the most sensitive stage of plant development for infection by *Fusarium*. In the period from 2008 to 2012 grains of wheat (cultivar Ritmo) from eight locations all over Schleswig-Holstein were analyzed for the occurrence of different *Fusarium* species and for their mycotoxin content. These results were combined with climate data which were present during the flowering stage of wheat every year. The samples were obtained from a monitoring program in cooperation with the Landwirtschaftskammer Schleswig-Holstein. Mycotoxins (A- and B-trichotheccenes, zearalenon) were determined by LC/MS and the presence of *Fusarium* species was analyzed by quantitative PCR.

Six different *Fusarium* species were detected from the wheat samples during the whole period: *F. graminearum*, *F. culmorum*, *F. poae*, *F. avenaceum*, *F. langsethiae* and *F. equiseti*. The B-trichotheccene synthesizing species *F. graminearum* and *F. culmorum* occurred in all years and at all locations as the most dominant species. The other species occurred at a clearly lower level and they were not even continuously present over all locations or years.

According to the dominance of B-trichotheccene synthesizing *Fusarium* species only deoxynivalenol (DON) and nivalenol (NIV) as well as zearalenone (ZEA) could be detected from all samples. A-trichotheccenes were not present. The highest amount of mycotoxins occurred in 2011 with a mean content of 2.13 mg/kg DON and 0.52 mg/kg ZEA. In 2009 and 2012 medium amounts with 1.05 mg/kg and 0.78 mg/kg DON and 0.16 mg/kg and 0.12 mg/kg ZEA could be detected. In 2008 and 2010 very low amounts with 0.08 mg/kg and 0.17 mg/kg DON and 0.01 mg/kg and 0.05 mg/kg ZEA were analyzed.

During the flowering stage in 2011 a precipitation of 36.3 mm and a mean temperature of 16.5 °C was present. In 2009 and 2012, the years with a medium amount of mycotoxins, 38.5 mm and 14.3 °C and 29.4 mm and 14.5 °C were present. The years 2008 and 2010 with the lowest amounts of mycotoxins had 3.5 mm and 17.5 °C and 7.3 mm and 14.9 °C. Thus high precipitation during flowering stage of wheat is essential for the occurrence of *Fusarium* mycotoxins. In contrast, the influence of temperature is not as strong, but also has an effect to the level of mycotoxin contamination of wheat.

These results clearly indicate a strong impact of climatic factors during the flowering stage of wheat to the contamination with *Fusarium* mycotoxins of the harvested wheat grains.
PRODUCTION AND OCCURRENCE – P52

Survey of Fusarium mycotoxins in grain and straw from Swedish pig farms

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Fusarium mycotoxins such as deoxynivalenol (DON) and zearalenone (ZON) are commonly found in various grain species in Europe. Recent studies indicate an increased level of contamination of grain in Sweden and Norway. Within EU maximum permitted levels in food are defined in legislation. For animal feed there are no legally binding maximum levels for DON or ZON however a maximum level of 8000 ppb in grain is recommended. Pigs are among the most sensitive species for those mycotoxins and clinical signs of adverse effects of DON have been observed at levels below 900 ppb.

In the present study a survey has been conducted where grain and straw were sampled at pig farms from different parts of Sweden during two years. Close to 100 samples were taken each year. The results show a certain variation between years, with DON almost ubiquitous in the samples from 2011 and in about 75% of the samples from 2012. Of the top ten levels of DON nine were from 2012, with a maximum content over 14 500 µg/kg in a sample of oats. The corresponding findings for ZON was six samples from 2012 and four from 2011 with a maximum content 1299 µg/kg of ZON in a straw sample from 2011.

Compared to grain much less attention has been directed towards straw as a possible source of mycotoxins Recently, Rohweder et al.\(^1\) showed that the bioavailability of DON in straw is comparable to that of DON in grain. In Swedish pig production straw is extensively used as bedding material, due to requirement in animal welfare legislation. Straw is also consumed in significant quantities by the pigs. More specifically, sows after weaning the piglets are often kept in flocks on restricted diet with a good supply of straw for bedding and leisure. These facts emphasise the importance of monitoring straw with respect to DON and ZON particularly when the straw is used in pig production.

References

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Distribution and toxigenicity of *Aspergillus* section *Flavi* in spices marketed in Morocco

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Characterization of fungal secondary metabolomes has become a challenge of great interest in the last decades due to the emergence of fungal threats to natural ecosystems and public health; and also due to Aflatoxin B1 is a carcinogenic mycotoxin that may contaminate spices. Indeed, these ingredients are usually produced in areas where the climatic conditions are favorable to growth of *Aspergillus* of the *Flavi* section and the synthesis of mycotoxins. This study aimed to characterize the fungal flora of 80 paprika, cumin and pepper marketed in Morocco with a special focus on *Aspergillus* section *Flavi* isolates. After identification to the species level, their toxigenic potential was determined and the related contamination of spice samples was also investigated.

We observed a widespread contamination of spices with *Aspergillus* section *Flavi*. Among them, 49% were found to be toxigenic. The most frequent chemotypes correspond to isolates able to produce both aflatoxin B and cyclopiazonic acid followed by B aflatoxins only producers. These chemotypes represented 44% and 27% of toxigenic isolates respectively. Ten percent of toxigenic isolates (3/29), that produced sclerotia in culture, were found able to synthesis aflatoxin G1. A molecular identification revealed that these isolates belonged to *A. minisclerotigenes* species. That is the first report of the presence of such species in spices.

The analysis of the contamination of spice samples with AFB1 revealed that paprika is frequently contaminated since 95% were contaminated with that mycotoxin and 40% of samples exceeded European regulation for that contaminant. Therefore, this spice that is widely used for cooking in Morocco, may represent a direct source of exposure to that carcinogenic compound.
Contamination level of *Fusarium* Mycotoxins, Fumonisin B1 and B2, in Animal Compound Feeds and Feed Ingredients in South Korea

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Fumonisins are mycotoxins produced by the grain molds such as *Fusarium moniliforme* and *F. proliferatum*. Optimum growth temperature of these *Fusarium* species is known to be 20 to 25 degree Celsius, but a wider range of growth temperature, was also reported in grain products from warm regions. The occurrence of Fumonisins in animal feeds can lead to a serious problem because these compounds may induce chronic or acute mycotoxicosis like “equin leukoencephalomalacia” and “porcine pulmonary edema” in animals and eventually humans.

In South Korea, grains such as rice, barley, wheat and maize are consumed as resources for major foods for human. In addition, some grains such as corn and soybean are also used as livestock feeds in many cases. Therefore, it is possible for human and animals to be exposed to some mycotoxins by intake of foods or feeds contaminated with mycotoxins producing fungi. From this point of view, the level of contamination of Fumonisin B1 (FB1) and Fumonisin B2 (FB2) was investigated in animal feeds included compound feeds and feed ingredients distributed in South Korea by using a modified analysis method of Korean Food Standards Codex and the law of AOAC.

The occurrence of FB1 and FB2 was investigated in 150 samples of compound feeds and 30 samples of feed ingredients that were distributed in South Korea in 2012. The contamination rates of compound feeds by FB1 and FB2 were 94 and 65.33 percent, respectively. In similar with this result, the feed ingredients exhibited contamination level of 83.33 and 50 percent for FB1 and FB2, respectively. The highest level of FBs was observed in the compound feeds for poultry (FB1: 100 percent and FB2: 72 percent), followed by cattle (FB1: 98 percent and FB2: 70 percent) and swine (FB1: 84 percent and FB2: 54 percent). However, a laying hen (the early period) feed showed the highest contamination value for FB1 (8343.35 nanogram per gram), and the level of FB2 was 9235.80 nanogram per gram in the early broiler feed which was identified as a maximally contaminated sample.

References


PRODUCTION AND OCCURRENCE – P55

Ochratoxin A – a risk in the supply chain of cereals

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Ochratoxin A is one of the most important toxic components with regards to food safety in cereals. This toxin is mainly produced by *P. verrucosum* after harvest during grain storage and it is known for its inhomogeneous distribution. For example, in a truck loaded with grain from the same field, one part of the lot can be free of ochratoxin A whereas another part can be highly affected with these mycotoxin hot-spots [1]. Such small, restricted areas with highly contaminated grains may cause an entire lot to violate legal limits [2]. Representative sampling is therefore an absolute prerequisite for the trueness of the analytical results [3]. The Regulation (EC) No. 401/2006 sets up universally valid sample taking procedures for officially governed mycotoxin contents in food. Depending on the kind of foodstuff and the size of a given sample lot, the pooling of up to 100 incremental samples to one aggregate sample is compulsory e.g. for a truckload. This constitutes a waste of resources and is hardly exercised in practice [4]. Instead a reduced number of random samples are taken which greatly increases the error of the analytical result. A real challenge in this context is the quality control of Ochratoxin A in dietary- and babyfood. The permitted legal limit within the European Union is currently at a level of 0.5 µg/kg in products intended for human consumption. Even in homogenized samples it could be shown that the standard deviation of results within the homogenate is still very high. Currently the possibilities to maintain this challenge are limited. One option is to higher the acceptance value of the uncertainty or bias of analytical results and to integrate this within the specification of the supply chain. Alternatively, it is still the best option to consider higher volumes of the raw materials for the mycotoxin analysis at the first step of processing to achieve more representative, reliable results.

References:
**PRODUCTION AND OCCURRENCE – P56**

*Worldwide occurrence of mycotoxins in feeds and ingredients in the year 2012*

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In 2012 a worldwide survey about the most important mycotoxins in feedstuffs was conducted to gather information on the presence of mycotoxins in commodities most commonly used for feed production. A total of 4,023 samples (mainly corn, soybean/soybean meal, wheat, dried distillers grains with solubles and finished feed) sourced in Americas, Europe and Asia were analysed for the presence of the following mycotoxins including aflatoxins (Afla), zearalenone (ZEN), deoxynivalenol (DON), fumonisins (FUM) and ochratoxin A (OTA).

Samples were analysed by high performance liquid chromatography (HPLC) and Enzyme-Linked Immunosorbent Assay (ELISA). Only single commodities were analysed by ELISA. More complex matrixes which could interfere with the ELISA method such as DDGS and finished feed were analysed by HPLC. For the purpose of data analysis, non-detect levels are based on the quantification limits (LOQ) of the test method for each toxin.

In the more than 4,000 samples analysed worldwide, Afla were present in 25%, ZEN in 46%, DON in 64%, FUM in 56% and OTA in 31%. Average contamination levels of all samples were 8 ppb for Afla, 115 ppb for ZEN, 696 ppb for DON, 759 ppb for FUM and 2 ppb for OTA. 18% were tested negative for the presence of five investigated mycotoxins. Thirty two percentages showed presence of one of them and two or more of the tested mycotoxins were present in half of all tested samples.

Results of this survey highlighted the necessity of mycotoxin testing prior to the feeding of animals. More than 80% were positive for at least one mycotoxin. The presence of more than one mycotoxin in half of the samples draws attention to the multi-mycotoxin contamination.
PRODUCTION AND OCCURRENCE – P57

Behaviour of mycotoxins during food processing

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The mycotoxins Deoxynivalenol (DON) and Zearalenone (ZON) are the most frequently occurring mycotoxins in German wheat produced by Fusarium spp. in the field. Also T-2/HT-2 toxins have been detected frequently in oats as well as Ergot Alkaloids (EA) in rye. Among others the European Union established maximum tolerable levels for some of these mycotoxins depending on the cereal production status to ensure consumer protection. However, risk assessment, natural occurrence, and hazard identification is still ongoing for these mycotoxins and in particular for their masked forms.

The aim of the presentation is to look at distribution profiles and the characteristics of the DON, ZON, T-2/HT-2, and EA content at different stages of the food production chain and particular processing steps in order to identify possible approaches to minimise the mycotoxin content in the final cereal based products.

Cleaning and sorting the grain material has an important influence on mycotoxin content. For DON e.g. up to 75 % decontamination rate seems to be possible with these steps in one batch. The remaining DON and ZON contents are located in different parts of the kernels. Whereas ZON content is elevated in the outer parts of the kernels, DON content is more evenly distributed within the whole kernel. Therefore, milling the grain and mixing together the different milling fractions has the potential to manage the mycotoxin content in the final flour to a certain extent.

T-2/HT-2 toxins are preferably located in the husk of the oat kernel and processing influences their content in different ways: cooking oat meal does not affect the mycotoxin content, whereas T-2/HT-2 concentrations were reduced after baking (Schwake-Anduschus et al. 2010).

The presentation will discuss influences of the baking process on DON, ZON, T-2/HT-2, and EA contents and the question to which extent masked mycotoxins occur and may contribute to the overall human mycotoxin exposure.

References
PRODUCTION AND OCCURRENCE – P58

Ochratoxin A analyses in edible offal and black sausages marketed in Belgium reveal only a small contribution to the total daily intake

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Pork derived products can contribute to the overall ochratoxin A (OTA) intake via carryover from contaminated feed or via mould spoilage of animal products (salami, dry-cured ham, sausage). To routinely monitor these foodstuffs for ensuring that the OTA levels are below the required limits, an UPLC/MS-MS method has been developed and validated to fulfill 2002/657/EC criteria. It offered detection limits of 93, 173 and 221 ng/kg and quantification limits of 200, 350 and 450 ng/kg for liver, kidney and black sausage, respectively. Recovery experiments carried out with spiked samples in the range of 5-10 µg/kg showed overall average recovery rates of 95% (RSDr=11%, liver), 108 (RSDr=11%, kidney) and 82% (RSDr=12%, black sausage) and maximal extended uncertainty (Umax, k=2) of 29%. Using the proposed maximal limits (ML) of 10 µg/kg for edible offal and 5 µg/kg (for black sausage), the decision limit (CCalpha) values were 12, 12 and 6 µg/kg whilst the detection capacity (CCbeta) values were 14, 13 and 7 µg/kg for liver, kidney and black sausage, respectively. The validated method was applied to the analysis of 20 kidneys, 20 livers and 20 sausages marketed during spring 2012 as edible offal in Belgium. Neither liver, nor black sausage samples were contaminated with OTA (<LOD). However, OTA was detected in 6 samples of kidney at mean value of 269 ± 134 ng/kg. The highest level found was 548 ng/kg, below the proposed EU limits. On the basis of the established tolerable daily intake (TDI) of 5 ng/kg body weight, accepted by the scientific committee on food of the EU, this study indicates that edible offal consumption in Belgium may contribute to less than 1% of the TDI based on the average consumption in Belgium. However, low OTA content may coexist with other derivatives (OTB, OTAalpha (glucuronide or sulfate form), OTbeta, open-ring OTA, 4-hydroxylated OTA, 10-hydroxylated OTA, OTQ (Quinone form) and OTHQ (hydroxyquinone)) that play a role in OTA toxicity. We therefore recommend checking the levels of other metabolites that may increase the OTA toxicity. As consumption of these pork derived products increases and generally not submitted to any compliance, it is worthwhile to further pay attention to the quality of feed.
PRODUCTION AND OCCURRENCE – P59

Incidence of mycotoxigenic fungi in wheat and maize from organic and conventional farming

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Organic farming is a growing system that is spreading worldwide in the last decades, but since it is characterised by the omission of synthetic pesticide usage, concerns about the incidence of mycotoxigenic fungi and the consequent accumulation of mycotoxins have risen.

In our study, we monitored the occurrence of fungi associated to organic wheat and maize from Italian fields, and compared it with conventional fields.

All samples were collected at the harvest in 2010 and in 2011. Relevant agronomic information were also collected. In particular, 195 samples of soft wheat and 29 of durum wheat were collected. Among them, 192 derived from organic and 32 from conventional farming. Also 66 maize samples were collected, 57 from organic and 9 from conventional system. Fungal incidence was measured in subsamples of 50 kernels plated on DCPA (SPELLING), then fungal identification was performed. Fusarium strains were identified at species level firstly based on morphological characters and therefore 10% of total samples were confirmed by molecular tools using a qualitative PCR analysis. Aspergillus and Penicillium spp. were identified at genus level based on morphological characters. Infected kernels represented 96% in both organic soft and durum wheat in 2010, but Fusarium infection was observed only in soft wheat (9% of kernels infected), and Aspergillus and Penicillium were almost absent. Fungal infection in both organic soft and durum wheat in 2011 was similar to 2010 (around 96%). However, while Fusarium presence was very low (0.8%), Aspergillus and Penicillium were more abundant, with the former more often isolated in durum wheat (25%).

Infected kernels in organic maize were 75% and 62% in 2010 and 2011 respectively, and Fusarium was frequently isolated (47% and 27%), while Aspergillus and Penicillium were almost absent as in organic soft wheat. On the other hand, the Fusarium occurrence in conventional maize corresponded only to 29% and 7.5% of infected kernels, in 2010 and 2011, respectively. The Fusarium species mainly recovered were: F. graminearum and F. poae in wheat; F. proliferatum, F. verticillioides and F. subglutinans in maize. As regard wheat, F. poae was the predominant species (43% up to 90% of Fusaria), except in organic soft wheat of 2010 where F. graminearum corresponded to 65% of Fusaria isolated. On maize, F. verticillioides was the dominant species (95%), followed by F. proliferatum (2%) in 2010, while the opposite was observed in 2011 (37% vs 52%, respectively). The other fungal species isolated from wheat and maize belonged to the Genera Epicoccum, Alternaria, Cladosporium, Rhizopus and Aureobasidium.

Finally, the total fungal infection was very similar in both organic farming and conventional growing system, while Fusarium infection was significantly different between the two systems, being in conventional wheat higher than in organic wheat especially in 2010 when the infection was around 12.5% of infected kernels). On the other hand, Fusarium infection in maize was significantly exacerbated in organic than in conventional farming system, showing an opposite behaviour of organic wheat and maize in their sensitivity to toxigenic fungus colonization.
The ability of cassava to inhibit Aflatoxin synthesis explains the absence of Aflatoxin contamination despite the presence of *Aspergillus flavus*

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Aflatoxin B1 is a carcinogenic mycotoxin produced by *Aspergillus* of the section *Flavi* that may contaminate many foods and feeds produced in tropical regions. Contamination with the mycotoxin may occur in the field or during storage. Cassava represents an important part of the diet of many people in sub-saharian Africa and both the culture and processing conditions may lead to its contamination by toxigenic fungal species and mycotoxins.

The first aim of this study was to evaluate the presence of aflatoxigenic fungi and aflatoxin B1 (AFB1) in cassava samples taken directly from producers. Thirty-six samples were analysed of which almost 40% were found to be contaminated by *Aspergillus* of the section *Flavi*. Morphological and molecular characterization by restriction fragment length polymorphism (RFLP) of the 16 isolates demonstrated that they belonged to *Aspergillus flavus* species. All these strains (as well as 12 others previously isolated from cassava samples taken at market level in Benin) were found to be toxigenic when cultured on potato dextrose agar. Nevertheless, no AFB1 contamination was observed in all the cassava samples. The second aim of this study was to investigate the action of cassava on AFB1 synthesis. For this purpose, a highly toxigenic strain isolated from cassava samples was inoculated onto fresh cassava and despite rapid development, the fungal isolate did not produce aflatoxin B1 on this substrate demonstrating that fresh cassava is able to block the mycotoxin production. This anti-aflatoxin property of cassava was lost after heating, sun drying and freezing. These results were confirmed using other aflatoxigenic strains of the section *Flavi* and two varieties of cassava from different geographical origins.

These results suggest that fresh cassava is safe regarding the danger of aflatoxin. However, processing may alter the ability of this raw material to block toxigenesis thus possibly leading to secondary contamination.
Diversity of *Alternaria* spp. from wheat by β-tubulin and glyceraldehyde-3-phosphate dehydrogenase gene sequencing and related mycotoxin production

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*Alternaria* spp. are the main causal agents of black point disease in cereals, particularly wheat and barley. Contamination of wheat kernels by *Alternaria* spp. has been reported in different countries including Italy. The disease consists in the discoloration of the germ-end of the kernel that can compromise grain quality and its commercial value especially if kernels are address to milling and processing pathway. Different *Alternaria* species have been associated to this disease, even though *A. alternata* is isolated with the higher frequency worldwide. *A. alternata* and other *Alternaria* spp. that commonly contaminate wheat, can produce toxic metabolites including alternariol, alternariol methyl ether, tenuazolic acid and altertoxins. Toxicity, mutagenicity and genotoxicity of these metabolites have been proven both in vitro and in vivo. Natural occurrence of these mycotoxins has been reported in wheat and grain based products worldwide representing a potential risk for animal and human health. Despite the ubiquitous presence of species included in this genus, *Alternaria* taxonomy is not completely clear yet, especially for the so-called “*Alternaria* small-spored species complex” that can be distinguished for few morphological characters. In addition, molecular phylogeny studies not always resulted reliable for species identification, when the most common genetic loci used for fungal phylogeny have been sequenced. In this study, two-hundred strains belonging to *Alternaria*, isolated from wheat kernels sampled worldwide were characterized by beta tubulin and glyceraldehyde-3-phosphate dehydrogenase gene sequencing. Molecular results grouped isolates in two main clades: the small-spored *Alternaria* species complex, including *A. alternata*, *A. tenuissima* and *A. arborescens*, and the *A. infectoria* species complex, that includes *A. infectoria* and *A. triticina*. Since each species can have its own mycotoxin profile, extracts of strains selected with respect to the geographic origin and the genetic clades will be analyzed by targeted and untargeted liquid chromatography - mass spectrometry for the presence of *Alternaria* toxins. The resulting data will be used for both define species boundaries according to a polyphasic approach, and establish more accurately the toxicological risks related to the specific contamination of wheat kernels and to set up control strategies for prevention.
PRODUCTION AND OCCURRENCE – P62

Assessment of mycotoxin exposure in the Belgian population using biomarkers

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Mycotoxins are important toxicological contaminants in our food chain, therefore it is relevant to estimate the exposure of our population to these toxins. Currently, the assessment of mycotoxin exposure is often based on calculations combining the occurrence in food with questionnaire data on food consumption. This indirect approach is not reliable due to the heterogeneous distribution in food, the exposure through inhalation, the presence of masked mycotoxins and the under- and overestimation in food consumption data. A more accurate assessment can be performed by direct measurements of biomarkers of exposure.

The purpose of this project is to perform an exposure assessment for a number of mycotoxins to which the Belgian population is exposed (mainly through dietary intake) by direct measurements of biomarkers of exposure. During the period January 2013 and January 2014 morning urine of 300 adults and 100 children will be gathered across Belgium. Moreover, a food frequency questionnaire was drawn up to assess the consumption of some relevant foodstuffs of both the day before the urine collection and the previous month. A general questionnaire will collect personal and socio-demographic data.

Aflatoxins, ochratoxin A, trichothecenes, zearalenone, fumonisins and their metabolites will be analyzed in the morning urine samples using a validated LC-MS/MS method. The obtained results will be normalized in function of the creatinin content. Correlations between the mycotoxin concentration measured and the foods consumption reported will be estimated, to explore whether the mycotoxin exposure can be explained by the consumption of certain foods.

Acknowledgements
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Recent occurrence of ochratoxin A in foodstuffs of plant and animal origin in Czech Republic - a preliminary results of a new research project

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Introduction:
Ochratoxin A (OTA) is a very important mycotoxin which is produced by Aspergillus and Penicillium species. OTA is a common contaminant of various foods including cereals, spices, coffee, cacao, beer, wine, raisins, pulses, meat, meat products or edible offal. In the Czech Republic, a new research project entitled "Ochratoxin A – health risk assessment for selected population groups in the Czech Republic" is taking place. The project objective is an assessment of dietary exposure and health risk characterization of OTA for ten population groups of age 4-90 years, both sexes. Grounds for decision of the project solution were recent knowledge about OTA carcinogenicity (adducts with DNA), cumulative effect of OTA in organism, an information about „large number“ of OTA exposure sources, recent results of urinary OTA excretion in men and women in CR and incomplete data of OTA dietary exposure in CR.

Methods for data collection and analysis
62 types of foods of plant and animal origin were collected in 4 sampling terms at 12 regions of the Czech Republic in the years 2011-2012. It represents 744 food samples. Validated and accredited ultratrace HPLC-FD method was employed for OTA determinations. The food samples were cleaned by means of immunoaffinity chromatography (OCHRAPREP® columns, R-Biopharm, Germany). Limit of quantification of the method (LOQ) was varied between 0.01-0.2 ng/g according to a type of sample matrix. The quality of laboratory results was confirmed by successful participation in international inter-laboratory proficiency testing (FAPAS, FERA, UK).

Results
The preliminary selected „higher“ analytical results (mean and range) concerning the occurrence of OTA in foodstuffs of plant and animal origin in hot red pepper (19 ng/g and 0.2 – 91.8 ng/g), sweet red pepper (16 ng/g and 0.2 – 38.4 ng/g), beans (9 ng/g and 0.2 – 107 ng/g), chilli (6.7 ng/g and 0.2 – 32.7 ng/g), coffee instant (1.04 ng/g and 0.2 – 4.91 ng/g), cocoa powder (0.94 ng/g and 0.2 – 4.10 ng/g), black pepper (0.83 ng/g and 0.2 – 2.82 ng/g), non-chocolate sweets (0.78 ng/g and 0.2 – 1.78 ng/g), biscuits (0.57 ng/g and 0.2 – 1.69 ng/g), raisins (0.46 ng/g and 0.2 – 2.17 ng/g), ginger bread (0.45 ng/g and 0.2 – 1.44 ng/g), coffee (0.41 ng/g and 0.2 – 1.04 ng/g), pork kidney (0.18 ng/g and 0.2 – 0.46 ng/g) were determined.

Conclusions
The analytical results will be served as a basis for an assessment of dietary exposure and health risk characterization of OTA for ten population groups of age 4-90 years, both sexes.

Acknowledgement
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PRODUCTION AND OCCURRENCE – P64

Gliotoxin producing isolates of *Aspergillus fumigatus* var. *ellipticus* distinguished by means of restriction fragment analysis

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This study focused on *Aspergillus fumigatus* isolates from silage. This saprotrophic and opportunistic pathogenic mould can cause spoilage of silage and can produce the mycotoxin gliotoxin (1-2). Although *A. fumigatus* is generally considered as a homogeneous species, strains may vary in both phenotypic and genotypic characteristics (3). In this study, variability of *A. fumigatus* isolates from silage was examined by pheno- and genotypic techniques and a rapid molecular method to distinguish different species/variants was developed.

Although molecular identification based on the Internal Transcribed Spacer (ITS) region indicated that all isolates concerned *A. fumigatus*, phylogenetic analysis showed that two clusters could be distinguished for these isolates from silage. In particular, one cluster contained *A. fumigatus* var. *ellipticus* isolates whereas the other cluster concerned *A. fumigatus* var. *fumigatus*. Remarkably, UHPLC-MS/MS analysis showed a statistically significant (*P*<0.05) higher in vitro gliotoxin production for *A. fumigatus* var. *ellipticus* isolates compared to *A. fumigatus* var. *fumigatus* isolates from silage.

Sequence analysis indicated the presence of a Single Nucleotide Polymorphism (SNP) at five positions in a fragment of the rodA gene (coding for a hydrophobin rodletA protein) between *A. fumigatus* var. *fumigatus* and *A. fumigatus* var. *ellipticus*. In order to distinguish these two types of isolates, a method was developed based on restriction analysis of this rodA gene fragment using the HinfI restriction enzyme. *In silico* analysis was performed to confirm the suitability of this method.

Combined with the earlier developed PCR Restriction Fragment Length Polymorphism (RFLP) method of Staab et al. (4), this method is part of a sequencing-independent identification scheme that allows rapid distinction between closely related species/variants within *Aspergillus* section *Fumigati*, specifically *A. fumigatus*, *A. fumigatus* var. *ellipticus*, *A. lentulus* Balajee & K.A. Marr, *Neosartorya pseudofischeri* S.W. Peterson and *N. udagawae* Y. Horie, Miyaji & Nishim. Seen the difference in gliotoxin production observed, this can be relevant from a toxicological point of view.

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References


PRODUCTION AND OCCURRENCE – P65

Contamination of fresh and ensiled maize silage by mycotoxins and mycotoxicogenic fungi

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Mycotoxins produced by Fusarium spp., Aspergillus spp., mycotoxicogenic species are reported in maize silage and have been associated with health problems in dairy cattle and mycotoxins in milk. The natural occurrence of mycotoxicogenic species and mycotoxins in maize silage has been reported by other researchers in Europe, this is the first study to determine contamination by mycotoxins in fresh and ensiled maize silage in Lithuania. The combination of PCR and ELISA has been used to assess maize silage contamination with fungi and mycotoxins.

The aims of this study were to estimate fresh and ensiled maize silage contamination with mycotoxins: deoxynivalenol (DON), T-2 toxin, zearalenone (ZEN) and aflatoxins total (AFL), and mycotoxicogenic fungi with PCR method with genes of the trichotecenes (TRI5; TRI6; TRI7; TRI13), aflatoxin (APA, OMT).

During 2011-2012 maize silage was collected from 20 farms in Lithuania. Samples were collected at harvest and the again at the same site 3 month after ensiling. The methods of dilution plating were applied for identification of fungi in samples of silage, before PCR method, were used microscopic examinations to identify the presence fungi until strains. DNA was extracted using Nucleospin Plant (Macherey-Nagel, Düren, Germany). PCR primers were designed from genes directly involved in mycotoxins biosynthesis, including trichotecene TRI5 (bp685), TRI6 (bp131), TRI7 (381-445bp), TRI13 (282bp), (2002; Schnerr et al., 2002; Chandler et al., 2003; Richard et al., 2009), aflatoxins biosynthesis APA (1034 bp) OMT (1254 bp) (Richard et al, 2009). Contamination of silage with DON, T-2 toxin, ZEN and AFL (total) was tested with the RIDASCREEN test kits (‘R-Biopharm AG’, Germany). In addition were collected chemical characteristics for each study site with method of Near-infrared spectroscopy. Fusarium species were detected in fresh maize silage. Aspergillus species – detected after 3 month of ensiled. Fusarium spp. – detected after 3 month of ensiled. Fusarium spp. – detected after 3 month of ensiled.

Fusarium sporotrichioides, F. poae, F. graminearum isolated from the different silage samples were positive for TRI5, TRI6, TRI7, TRI13. Aspergillus flavus were positive for APA and negative for OMT, other Aspergillus spp. – positive for OMT and negative for APA. The mean concentrations of four mycotoxins detected in fresh and ensiled maize samples, respectively: DON (1640.0±39.0 µg/kg and 2600.0±26.0 µg/kg); T-2 (40.21±18.24 µg/kg and 141.47±23.46 µg/kg); ZEN (199.7±29.05 µg/kg and 880.4±60.62 µg/kg), AFL (total) (0.94±0.52 µg/kg and 16.86±3.96 µg/kg). Compared fresh and maize silage samples, higher mycotoxins concentrations were detected in samples after ensilage: DON – 36.96% (p>0.05), T-2 toxin – 71.58% (p<0.05), ZEN – 77.32% (p<0.05), AFL (total) – 94.42% (p<0.05).

The presence of DON, T-2 toxins, AFL (total) in the maize silages samples can be compared to the positive PCR with primers specifically targeted TRI5, TRI6, TRI7, TRI13 and APA, OMT genes, respectively. Our study developed the presence of potentially mycotoxicogenic Fusarium spp. in fresh maize silage and Aspergillus spp. in ensiled silage. Higher trichotecenes concentration after ensilage showed its stability during ensiling process. Data from this study develop what mycotoxins and mycotoxicogenic fungi may be expected in Lithuanian maize silage and required further research for their detection.

References
PRODUCTION AND OCCURRENCE – P66

Study on analytical methodology and in vivo kinetics of the typical mycotoxins in traditional Chinese medicines

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A generic procedure, which involved accelerated solvent extraction (ASE) and home-made clean-up cartridges, was developed for the extraction and purification of 35 mycotoxins in various traditional Chinese medicine (TCM) matrices, i.e., rhizomes and roots, seeds, flowers, grasses and leaves, for subsequent analysis by liquid chromatography tandem mass spectrometry (LC-MS/MS). All target analytes could be simultaneously quantified in less than 17 min per run, showing narrow symmetrical peaks. The developed method was also successfully applied in routine monitoring programs, which implied a significant reduction of both effort and time, to investigate the contamination of TCMs. Among 60 commercial TCMs analysis, 50 were positive. Based on the screening results and the regulation limits, aflatoxin B1 (AFB1), ochratoxin A (OTA) and T-2 toxin (T-2) were selected for the in vivo kinetics and biotransformation study in rat. A combinatorial approach of LC-MS/MS and LC-TOF-MS was developed for quantitative and qualitative analysis of AFB1, T-2, OTA and their metabolites in plasma, heart, liver, spleen, lung, kidney and brain in rat. As revealed, both AFB1 and T-2 were rapidly eliminated with a half-life time (t1/2) in plasma of 8.44±4.02 h and 8.12±4.05 h, respectively, while a very slow elimination was observed in OTA with a t1/2 of 75.6±29.0 h. Results of tissue accumulation showed that AFB1 accumulated in all organs where the highest concentration was observed in liver (1.34 μg kg⁻¹), followed by kidney (0.76 μg kg⁻¹) and the highest concentration of OTA was observed in lung (95.9 ± 13.7 μg kg⁻¹), followed by liver (76.0 ± 9.7 μg kg⁻¹), heart (62.0 ± 4.2 μg kg⁻¹) and kidney (55.7 ± 4.7 μg kg⁻¹). Notably, only low levels of T-2 were observed in spleen (0.70 μg kg⁻¹) and in liver (0.15 μg kg⁻¹). Furthermore, three less toxic metabolites of OTA were clearly identified: ochratoxin β (OTβ) and ochratoxin B (OTB) methyl ester were found in kidney and spleen, respectively, while phenylalanine was detected in heart and kidney.

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Validation of a multiplex microsphere immunoassay for mycotoxins in barley and confirmation of results for global beer samples

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Beer is one of the oldest alcoholic beverages known in the world. With a total global beer consumption of 180 million liters in 2010, beer became the most popular alcoholic beverage. Just a small part of the total beer consumption concerns craft beers. Nowadays however, craft beer is booming and is often referred to as “the new wine”. This is mainly based on the creativity and a whole range of new beer styles that come with it. Regardless of the style, the main ingredients of beer generally are; water, barley, hops and yeast. Barley, and to a lesser extent hops, can be contaminated with mycotoxins. During steeping, germination and kilning contaminating fungi are still able to grow and produce mycotoxins. This is also the process where seedlings conjugate DON to the masked form deoxynivalenol-3-glucose (DON3G) which will most likely not be detected by routine analysis.

In the present work, we validated a fast direct microsphere immunoassay (MIA) for multiplex screening of the mycotoxins; aflatoxin B1 (AFB1), fumonisin B1 (FB1), deoxynivalenol (DON), ochratoxin A (OTA), zearalenone (ZEA) and T-2 toxin (T-2) in barley. This was done according to the Commission Decision (2002/657/EC) for screening assays. The assay was developed using Luminex’s multi-analyte profiling (xMAP®) technology featuring paramagnetic color-encoded microspheres. The mycotoxin specific monoclonal antibodies were coupled to the microspheres. For the detection we created unique reporter molecules by the conjugation of the aforementioned mycotoxins to R-Phycoerythrin. For assay validation we used a MAGPIX® machine which is capable of measuring dedicated paramagnetic microspheres in planar array format. To make this lab-assay more suitable for on-site testing, we replaced pipets with droplet bottles and also tried to eliminate as much laboratory equipment as possible from the method. Also this droplet-assay was subjected to the screening assay validation.

Furthermore, with the lab-assay a total of 815 international beer samples were screened covering 58 beer styles originating from 36 counties of which 45% were craft-brews. Based on the MIA results we made a selection of 170 beers based on contamination, style and origin, for confirmation by a multi-mycotoxin LC-MS/MS method. For this a dedicated multi-LC-MS/MS method was developed that detects the same 6 mycotoxins, as well as their available metabolites and masked forms. As expected DON and DON3G were the main contaminants in most beers. In some beers we found DON (sometimes combined with DON3G) which exceeded the tolerable daily intake. In some cases the level of DON3G exceeded the level of DON. In Southern European adjunct beers and South African home-brewed beers we found FB1 with a maximum concentration of 50 ng/ml. Not in all cases, especially for AFB1, the MIA data could be confirmed by the multi-LC-MS/MS method. Therefore a selection of beer samples with inconclusive results will be further investigated. using bioaffinity-based identification approaches.
Study of D3G occurrence in greenhouse experiment

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In recent years the incidence of Fusarium related diseases such as Fusarium Head Blight (FHB) in crops is increasing, causing extensive losses in wheat production and severe toxicological problems due to mycotoxin accumulation. Research has thus moved on the study of plant genotypes having the genetic ability to resist to Fusarium related disease. Among cereals, soft wheat and barley have been extensively studied, demonstrating that the in field resistance toward FH is often related to the ability of convert deoxynivalenol and other Fusarium toxins to their masked forms (e.g. conjugates with glucose, glutathione or sulphate). Low attention has been given to minor crops such as durum wheat or small grains. Durum wheat is a key crop in Italy, where pasta production is totally based on this cereal. In addition, durum wheat based production also includes several high quality bakery products such as IGP/PDO breads. In this study, we present some preliminary results about the ability of four different durum wheat lines to convert DON to deoxynivalenol-3-glucoside upon fungal inoculation or DON contamination under greenhouse controlled conditions. In particular, three groups have been considered: plants treated with F. graminearum, plants treated with DON and control plants. Both inoculation and contamination were made on the flowering ears and induced typical FHB symptoms to the plant. Plant ears were sampled at different times from the inoculation and analysed for DON and D3G occurrence. Data were statistically analysed and compared to those obtained for control plants. Results demonstrated that the ability to convert DON to its masked forms is genotype-related also in durum wheat.

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References
Occurrence of Fusarium Mycotoxin, Deoxynivalenol, in Animal Feeds in South Korea

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Introduction: DON is produced by strains of Fusarium graminearum (Miller et al., 1983) or F. culmorum (Greenhalgh et al., 1986), which are common pathogens of cereals. Recently, the occurrence of mycotoxins in feeds has become a major concern because these compounds may be presented in foods and can be resulted in chronic or acute mycotoxicosis in animals and humans. Contamination by DON is expected to increase with global warming. This survey aimed to determine the occurrence and level of DON and to increase food safety by understanding the contamination of feed by DON in South Korea.

Material and Methods: Feed samples were milled (particle size, 600 μm) and weighed to the nearest 0.1 gram and 20 gram of each sample was extracted with 100 milliliters of extraction solvent (100 percent water) in a homogenizer for 2 minutes (10,000 rpm). The extract was filtered through Whatman filter paper. Filtered extract was applied to the immune affinity column (IAC: Vicam, Don test). The IAC column was washed 5 milliliters of water, and then DON was eluted from the column with 3 milliliters of 100 percent methanol at 1 drop per second. The eluted fraction was evaporated to dryness for analysis by high-performance liquid chromatography (HPLC).

Results: The contamination of feed ingredients by DON was 91 percent. All samples of the cattle feeds were contaminated with DON. 53 percent of the feed ingredient samples were contaminated by DON with a mean concentration of 332.70 nanogram per gram. 74 percent of the swine feeds were contaminated with DON with a mean value of 121.46 nanogram per gram. 78 percent of the poultry feeds were contaminated with DON with a mean concentration of 212.63 nanogram per gram.

Summary: The occurrence of DON was investigated in 150 samples of compound feeds and 30 samples of single ingredients that were distributed in South Korea in 2012. 163 samples were contaminated with DON in feeds of 180 samples. The highest level of DON was observed in compound feeds for cattle (DON: 100 percent), followed by poultry (DON: 96 percent), swine (DON: 92 percent) and single ingredients (DON: 80 percent). However, Layer Chicken feed exhibited the highest contamination value (1175.2 nanogram per gram) for DON.

Reference:
PRODUCTION AND OCCURRENCE – P70

Alternaria Toxins – Background and Analytics

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Different food and feedstuffs can be affected by molds and could therefore contain mycotoxins endangering human or animal health. These molds include the genus Alternaria (black molds) which preferably infects cereals, vegetables and fruits. Depending on the species and growing conditions a variety of toxins can be produced and might remain in the corresponding food or feed.

In 2003, the BfR evaluated Alternaria toxins in foodstuffs with regard to a potential risk for human health. The current scientific opinion expressed most recently by EFSA in 2011 is that the black molds can also produce mycotoxins which cause diverse affects and could therefore be a threat for the health of animals and humans. However, until now the chemical structure of approximately 30 Alternaria toxins is known and only for 7 toxins toxicological data are available.

At present, the database concerning the occurrence of Alternaria toxins in foodstuff is very limited. So, the exposition of consumers by Alternaria toxins can only be hardly assessed.

Regarding feedstuff the situation is comparable, especially concerning grass and maize silages. How far animal health is affected by Alternaria toxins and to what extent Alternaria toxins are transferred into products of animal origin are only some open questions of this group of mycotoxins in the risk assessment.

The analysis of Alternaria toxins is currently lacking commercially available standard substances in some instances and standardised validated analytical methods. A reliable alternaria mycotoxin analysis method must be developed for tracing their source in the food production chain. Some analytical aspects to the development of a multi-analyte method for the liquid chromatography-tandem mass spectrometric determination of Alternaria toxins will be discussed.

References
### PRODUCTION AND OCCURRENCE – P71

#### Phytotoxin production by *Cochliobolus miyabeanus*, the causal agent of brown spot disease on rice

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Plantpathogenic fungi are a constant threat to living plants. When they cause disease on important crops, they can result in enormous economic losses and affect the food security of many people. For instance, an outbreak of rice brown spot disease caused by *Cochliobolus miyabeanus* (anamorph *Bipolaris oryzae*) in 1942 in rice fields (*Oryza sativa*) in India resulted in the Bengal Famine, making 2 million casualties. *C. miyabeanus* is a necrotrophic phytotoxin producing fungus. Like many other *Cochliobolus* species, *C. miyabeanus* produces multiple ophiobolins, a family of sesterterpenoid non-specific toxins. Many of the known phytopathogenic fungi belonging to the same genus produce an additional host-specific phytotoxin. However, for *C. miyabeanus* no host-specific toxin has been identified yet. And besides the already described ophiobolins, no other non-specific phytotoxic compounds are known. Phytotoxins are secondary metabolites that typically kill the plant cell by interacting with important plant biochemical pathways instead of causing severe damage. Evidence suggests that the interaction between a phytotoxin and its respective plant target induces programmed cell death; in other words, the fungus actually reprograms the plant cell to commit suicide. It is our aim to identify new, possibly host-specific, phytotoxins produced by *C. miyabeanus*.

In a first part of the research we are trying to identify and characterize new phytotoxic compounds in a *C. miyabeanus* culture filtrate. An extract of the filtrate is separated by UPLC on a C18 column and different collected fractions are tested for bioactivity against rice, using rice leaf bioassays as well as rice cell cultures. The extract is further analyzed by UPLC- high resolution mass spectrometry, and accurate mass and fragmentation data are used to identify the compounds in interesting fractions. Additionally we compare the toxin production of highly and weakly virulent strains to identify which secondary metabolites are most important for virulence. In a second part of the research we try to define the role of different produced toxins (ophiobolins, as well as newly identified) in the infection process. We investigate what kind of cell death is induced and which signalling pathways might be targeted by the toxins.
A limited survey of mycotoxins in malted maize and traditional maize based opaque beers in Malawi

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A total of 9 traditional maize based opaque beer and 5 malted maize samples were randomly collected from tribal (chewa) rituals and commercial village brewers from Lilongwe and Dowa districts, Malawi in August 2012 for evaluation of mycotoxins using a multi-mycotoxin method based on liquid chromatography-tandem mass spectrometry. Samples were analysed for aflatoxins B1 (AFB1), AFB2, AFG1, AFG2, fumonisin B1 (FB1), FB2, FB3, zearalenone, ochratoxin A, nivalenol, deoxynivalenol, 3-acetyldioxynivalenol, 15-acetyldioxynivalenol, neosolaniol, fusarenon-X diacetoxyisirpenol, HT-2 toxin, T-2 toxin, alternariol, alternariol, methyl ether, altenuene, mycophenolic acid, sterigmatocystin and beauvericin. All beer and malt samples were positive for fumonisins with a total fumonisin (FB1+FB2+FB3) concentration of 1900±1400 μg/kg (mean±stdev) and 42000±27900 μg/kg respectively. With exception of one beer sample, all the beer and malts also contained aflatoxins at a mean concentration of 90±95 μg/kg and 180±200 μg/kg respectively. Nivalenol and deoxynivalenol were only detected in 4 and 3 of the malt samples at a mean concentration of 150±80 μg/kg and 160±140 μg/kg respectively. Additionally altenuene was detected in two beer samples and mycophenolic acid and beauvericin were each present in a single beer samples. Thus consumption of the opaque maize-based traditional beers poses a risk of multiple mycotoxin exposure.
PRODUCTION AND OCCURRENCE – P73

Occurrence of toxigenic fungi, aflatoxins and ochratoxin A in wheat, rice, dried fruits, and spices commercialized in Algeria

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The aim of this study was to investigate the presence of toxigenic fungi, aflatoxins (AFs) and ochratoxin A (OTA) from local wheat, rice dried fruits, and spices form Algeria. A total of 284 samples (108 wheat, 50 spices, 96 dried fruits, 30 rice) were analyzed. The wheat samples were collected during pre-harvest, storage in silos and after processing. Studies on mycobiota make it clear that *Aspergillus* species are frequent in these commodities, sections *Flavi* and *Nigri* being the predominant aspergilla. Among isolates of *Aspergillus* section *Flavi* examined, 45% produced high levels of AFs. The most aflatoxigenic strains (90.6%) were identified as *A. flavus*. Aflatoxin B1 (AFB1) was detected by HPLC in 56.6% of the wheat samples with contamination levels ranging from 0.13 to 37.42 μg/kg, and in 90% of the dried fruits with contamination levels ranging from 0.16 to 25.82 μg/kg. This mycotoxin was detected in 13 of 21 (61.9%) samples of spices with quantities ranging from 0.1 to 24.3 μg/kg. OTA was detected in 12 (40%) of the wheat samples at levels ranging from 0.21 to 41.55 μg/kg.
PRODUCTION AND OCCURRENCE – P74

French survey of occupational exposure to mycotoxins: biomarkers and airborne contamination measurements

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Ingestion of mycotoxins from contaminated food products is deemed to constitute the main important source of exposure in the general population. There is also today a growing interest in the role of mycotoxins as health hazards in the workplace. Mycotoxins have been identified in various occupational environments including poultry productions, agricultural and food processing facilities, livestock feed productions, indicating that inhalation and dermal contact may represent an additional route of exposure. To what extent such exposure results in potential risks for health for these workers remains unclear.

In order to obtain some data about occupational exposure to mycotoxins, the french occupational safety and health institute INRS sets up a project to assess external and internal exposure to some mycotoxins in various workplaces with a focus on food and feed processing facilities, livestock and poultry farming. For external exposure, airborne contamination will be determined by personal and ambient air sampling. To investigate the respiratory and dermal intake, human biomonitoring of mycotoxins from a cohort of workers will be implemented. Ochratoxin A, aflatoxin B1, fumonisin, zearalenone, deoxynivalenol and their metabolites will be determined in urine using a multimycotoxin method by LC-MS/MS. For an interpretation of human biomonitoring data, the results will be compared with those obtained from non-occupationally exposed persons and the relation between airborne contamination and measured biomarkers will be examined.

This survey, which will take place between 2014 and 2016, would allow the mapping of mycotoxins occurrence in occupational settings and further progress in assessing mycotoxins health impact in some typical occupational environments.
PRODUCTION AND OCCURRENCE – P75

Mycotoxins of endophytic *Fusarium mangiferae* and *F. pallidoroseum* from betel leaves (*Piper betle* L.)

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Betel (*Piper betle* L.) is a tropical perennial evergreen climber growing in Asia. The fresh leaves are consumed by people of Laos, Kumpuchea, Malaysia, Myanmar, Thailand, Singapore, and the Indian subcontinent. Betel leaf is chewed as a quid which is made by rolling slaked lime and betel nut (Fig. 1), with or without tobacco in fresh betel leaves. An estimate in the year 2006 indicates that in India alone about 55,000 hectares were under betel cultivation and about 15 to 20 million people consume betel leaf. Betel leaf is known for its medicinal properties as it has antioxidant, antiinfective, analgesic, anticancer, antidiabetic, hepatoprotective and immuno-modulatory properties. Considering these aspects and the wide-spread consumption of betel leaves in South-East Asia, we decided to survey *Piper betle* for colonization by endophytic fungi.

*Fusarium* endophytes isolated from betel were assigned to *F. mangiferae* and *F. pallidoroseum* by morphology and EF1a and ITS of rDNA sequences. Mycotoxin production by these species has not been thoroughly studied. The analysis of rice cultures by HPLC-MS/MS showed that *F. mangiferae* produced large amounts of nivalenol, fusarenon X and equisetin. *F. pallidoroseum* produced large amounts of beauvericin and trace amounts of enniatin A.

The presence of mycotoxin-producing *Fusarium* spp. indicates a potential health risk due to chewing betel leaves. We intend to survey betel on the market for mycotoxin content.
PRODUCTION AND OCCURRENCE – P76

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

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The secondary metabolites AOH and AME are two naturally occurring mycotoxins formed by *Alternaria alternata* and have been detected to be widespread contaminants of food and feed all over the world, e.g. fruits, but also processed fruit products (Ostry, 2008). Genotoxic and mutagenic properties of AOH and AME in mammalian cells in vitro have been reported, e.g. AOH was reported to act as a topoisomerase poison (Fehr et al., 2009). Phase I metabolites (Figure 1) of AOH and AME have been detected and identified but not synthesised for further studies or as reference materials so far. These compounds are formed by monohydroxylation of AOH and AME, a prominent metabolic route under cell-free conditions (Pfeiffer et al., 2007).

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

![Scheme 1: Key steps of retrosynthetic analysis of target compounds; R = H (AOH) or Me (AME)](image)

All aromatic building blocks (benzoic acids and substituted phenols) for monohydroxylated metabolites of AOH and AME were synthesised. The first key step, esterification of acid and phenol building blocks, was performed for all metabolites. C-H activation of the obtained esters was first carried out to yield the 4-hydroxylated intermediate. Selective deprotection yielded the first synthetic phase I metabolites 4-hydroxy-AOH and 4-hydroxy-AME. These compounds were submitted for toxicological analysis and serve as reference materials for further investigations. The preparation of 2-, 8- and 10-hydroxylated compounds is subject of on-going research, which will be presented and updated in this poster presentation.

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### Fungi and its mycotoxins on organic winter rye after artificial infection by *Fusarium* spp.

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Especially dangerous for grain quality of cereals is *Fusarium* head blight. Most common species caused FHB are: *F. culmorum*, *F. graminearum*, *F. nivale*, *F. poae* and *F. avenaceum*. This fungi are present in all climatic zones. Occurrence of *Fusarium* diseases can be highly variable. On limited area can coexist toxigenic fungi vary according to intensity of spores production, infection efficacy and growth rate. Also spectrum of mycotoxins produced by each species can be different according to field conditions and other organisms presence.

During all period of disease occurrence in cereals from first literature report till today composition of *Fusarium* species responsible for FHB was changed. First information about *Fusarium* spp. infestation was from Canada from 1950 and species responsible for FHB were: *F. avenaceum*, *F. culmorum*, *F. graminearum*, *F. poae* and *F. sporotrichioides* [Panisson et al. 2003]. Nowadays in rye grain most common mycotoxins are deoxynivalenol, nivalenol and fumonizins produced by: *Fusarium moniliforme*, *F. proliferatum*, *F. graminearum*, *F. culmorum*, *F. crookwellense*, *F. sporotrichioides*, *F. poae*, *F. acuminatum* [Clifford et al. 2003]. Kiecana and Mieleniczuł [2010] found that in Poland *F. avenaceum*, *F. culmorum* and *F. sporotrichioides* are most dangerous for winter rye. According to present research winter rye cultivars have vary resistance for *Fusarium* diseases.

The aim of this study was to determine the presence of toxigenic fungi and produced by them mycotoxins in three organic winter rye cultivars growing in Poland after artificial infection by *Fusarium poae* and *F. sporotrichioides* isolates. Conventional mycological analysis was used for the detection of fungi especially *Fusarium* species in rye grain. The occurrence of mycotoxins as aflatoxins, ochratoxin A, T-2 toxin, deoxynivalenol and zearalenone was defined by the Enzyme-Linked ImmunoSorbent Assay (ELISA).

On winter rye kernels dominates fungi from *Alternaria* genus, second were *Fusarium* spp. and third *Penicillium* spp.. Among *Fusarium* spp. mostly *F. culmorum*, *F. graminearum*, *F. poae*, *F. avenaceum*, *F. sporotrichioides* and *F. equiseti* were isolated from rye kernels. Most numerous were *Fusarium sporotrichioides* isolates – 44%, second *F. culmorum*, *F. avenaceum* and *F. equiseti* – equally 11%, third *Fusarium graminearum* – 7%. The less numerous were *F. poae* isolates – only 5% and *F. oxysporum* – 1%. In all winter rye cultivars ochratoxin A, deoxynivalenol, zearalenon and T-2 toxin were identified but in all investigated samples aflatoxins were not found. Rye cultivars were mostly contaminated by T-2 toxin up to 256,89 µg/kg. In grain also zearalenon in high amounts up to 609,78 µg/kg was found. OTA and deoxynivalenol were identified in almost all samples but in amounts not excided tolerated level.

### References


**Mycological profile of mouldy pineapple from Czech retail**

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**Introduction:**
Pineapple (*Ananas comosus* (L.) Merr.) is native to South America and widely planted as a fruit crop in the tropics and sub-tropics (e.g. in middle and south Africa). This plant is susceptible to a number of fungal diseases of which the most severe is fusariosis and fruitlet core rot (*Penicillium funiculosum*). Fungal diseases caused by six organisms were recorded, namely: *Botryodiplodia* sp., *Aspergillus niger*, *Penicillium digitatum*, *Fusarium* spp. and *Rhizopus stolonifer*. The pathogenicity of each of the organisms was established. The strong virulence caused by *Botryodiplodia* sp. and *Aspergillus niger* during pathogenicity tests clearly suggested *Botryodiplodia* sp. and *Aspergillus niger* as the most important fungal disease pathogens of pineapple fruits in Nigeria. The recent informations about mycological profile of pineapples and an occurrence of toxigenic microfungi in pineapples after harvest are scarce.

**Methods for data collection and analysis**
One mouldy pineapple was sampled from mouldy pineapple consignment in Czech retail. The country of origin of pineapples was the Ivory Coast.

Isolates of microfungi are usually isolated and characterized on nutrition media e.g. PDA, MEA and CYA. The strains of microfungi were studied in culture and identified by FoodMold (*an interactive CD guide to the most common foodborne filamentous fungi / moulds/). The modified, validated and accredited LC-MS/MS method was employed for purposes of the determination of nine trichothecenes A and B (DON, 3-AcDON, 15-AcDON, DON-3-Glu, NIV, T-2 toxin, HT-2 toxin, DAS, FUS-X) and ZEA. The pineapple sample was extracted with acetonitrile/water (84:16, v/v). Swift clean-up of pineapple samples was performed with MycoSep 226 column. The limits of quantification (LOQ) of the method were between 0.05-1.7 ng/g.

**Results**
*Fusarium* spp. and *Penicillium digitatum* were isolated and identified from the sample of mouldy pineapple. *Fusarium* spp. is a large genus of microfungi widely distributed in soil and in association with plants. Some *Fusarium* species produce mycotoxins (e.g. trichothecenes, zearalenone and fumonisins). *Penicillium digitatum* is a plant pathogen. It is a common postharvest fungal disease of citrus called “green mould”. At present, nothing is known regarding the toxigenity of *Penicillium digitatum*. *Penicillium funiculosum*, *Aspergillus niger*, *Fusarium ananatum*, *F. guttiforme* and *F. verticillioides* were not isolated. The results of the determination of nine trichothecenes and zearalenone in pineapple were under the limit of quantification (LOQ) of the method.

**Conclusions**
Results of this study have shown that we need further relevant data about the occurrence of toxigenic microfungi in pineapples after harvest in retail.

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Impact of cultivation and fertilization on mycotoxins occurrence in winter wheat

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Fusarium head blight is a serious disease of small grain cereals caused severe and repeated epidemics resulting in enormous losses[1, 3]. Many of Fusarium species are capable of producing mycotoxins, which can cause a wide range of acute and chronic effects in humans and animals through food and feed prepared from contaminated grain[4]. The Fusarium species predominantly found associated with Fusarium head blight in wheat and other small-grain cereals all over Europe are F. graminearum, F. avenaceum and F. culmorum. Among the less frequently encountered species are several others which are less pathogenic or opportunistic, but also toxigenic. These include F. poae, F. cerealis, F. equiseti, F. sporotrichioides, F. tricinctum and to a lesser extent F. avenaceum, F. solani, F. oxysporum, F. moniliforme. The species profile of Fusarium head blight is due to several factors, primarily climatic conditions, particularly rain and the temperature at flowering stage, but also agronomic factors, such as soil cultivation, nitrogen fertilization, fungicides, crop rotation, and host genotype [2].

The aim of this study was the identification of fungi and determination of mycotoxins content in the grain of winter wheat. The studied material was the grain of two selected cultivars of winter wheat, Muszelka and Tonacja with and without undersown clover.

The main cause of Fusarium head blight of winter wheat cv. Muszelka grown without undersown was F. avenaceum and F. poae was main pathogen of both cultivars with undersown clover. The associated fungi in causing of the disease were F. equiseti, F. sporotrichioides, F. graminearum and F. tricinctum.

Occurrence of fungi on the grain winter wheat depended on the variety and the way of cultivation. Growing of winter wheat with undersown clover resulted in decrease the number of fungi of Fusarium in grain of cv. Tonacja by 7%, but in increase the number of these fungi by 16% in case of cv. Muszelka compared to growing the cultivars without undersown.

Highest share amongst isolated fungi from winter wheat grains constituted Alternaria alternata. There was no relationship between the number of fungi of the genus Fusarium and the mycotoxins content in grain. The winter wheat grain contamination by mycotoxins was influenced by cultivar and a way of growing of the crop.

Growing of winter wheat with undersown clover resulted in decrease of T-2 toxin, but in increase of deoxynivalenol and zearalenone in a grain of both cultivars.

References
PRODUCTION AND OCCURRENCE – P80

How do *Fusarium verticillioides* and *Fusarium graminearum* interact when grown on artificial media and maize cobs?

Ellner, F.M.

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*Fusarium graminearum* Schwabe (telemorph *Gibberella zeae* (Schwein.) Petch) and *Fusarium verticillioides* (Sacc.) Nirenb. often co-occur on maize plants showing pink and red ear rot symptoms. Both diseases are accompanied by a mycotoxin contamination in the kernels. However, there are contradictory reports concerning the interactions between *F. graminearum* and *F. verticillioides* in terms of growth and mycotoxin production in both artificially-infected maize kernels and pure cultures.

To understand the consequences of these interactions we investigated growth behaviour and mycotoxin production of both fungi cultivated on different artificial media and maize cobs under various experimental designs.

Having been cultivated on the same agar plate, both fungi showed undisturbed growth rates on all media tested. Neither any inhibition zones nor growth retardation of either candidate could be detected even in the regions were the fungi overgrew each other. Mycotoxin production depended on the inoculation scheme characterized by placing agar discs from pre-cultures of the corresponding fungi side by side either in short or long distances. We observed an induction of trichothecene production of *F. graminearum* by *F. verticillioides* and a reduction of fumonisin production the other way round. Using maize cobs, harvested at dough stage, as a substrate similar results were found. When both fungi were cultivated on the same cob deoxynivalenol concentrations increased and fumonisin B1 and B2 production was reduced. Both fungi grew absolutely unimpaired from each other on maize cobs even in the overgrowing zones. Affects on mycotoxin production increased the closer the growing zones came in contact with each other.
PRODUCTION AND OCCURRENCE – P81

Fusarium species and mycotoxins in weed plants

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Organically and conventionally grown maize contain similar levels of mycotoxins on the average, but extremely high levels have occasionally been reported in organic maize. Field trials lead to similar results (Nutz and Karlovsky, unpublished). Weed plants infected with Fusarium may account for this phenomenon. 295 samples of 56 weed species collected from organic and conventional maize fields were examined for the presence of F. graminearum, F. verticillioides, F. culmorum, F. subglutinans, F. poae, F. avenaceum and F. proliferatum by PCR. Some Fusarium species were detected with very high frequencies (Fig. 1).

Figure 1: Colonization of weed plants in maize fields by Fusarium species

Because F. avenaceum was found in 35% of all samples, enniatin content was determined in weed samples by HPLC-MS/MS. Enniatin B was found at high amounts in Chenopodium album (24 mg/kg), Elymus repens (11 mg/kg) and Viola arvensis (7 mg/kg). Beauvericin (e.g., 4 mg/kg in Chenopodium album) and other mycotoxins were found, too. Eighty five Fusarium isolates were recovered from weed samples and characterized morphologically and by sequencing selected genes. Re-inoculation experiments with 12 weed species showed that each weed species was susceptible to colonization with several Fusarium species.
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Detection of moulds in patients with chronic rhinosinusitis

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Among the most common etiological factors contributing to sinusitis are: anatomic abnormalities, environment pollution, bacteria, viruses and fungi. Fungal infections are often mistakenly diagnosed as bacterial ones, thus making antibiotic therapy ineffective.

In the years 2011-2012 mycological tests of the biological material, taken from patients operated due to chronic rhinosinusitis in ENT Department at University Hospital No 2 in Bydgoszcz, were carried out. Fragment of mucous membrane from around the ethmoid bulla was taken during the surgery, because its microbial characteristics is the most similar to that of mucous membrane of nasal sinuses lumen. Moreover, nasal lavage fluid was also collected.

The tissue material was incubated in liquid medium (Sabouraud 2%) for 24 h and then the suspension of no more than 1ml each was sieved on the surface of various media: MEA (Malt Extract Agar), IMA (Inhibitory Mold Agar) and BHIA (Brain Heart Infusion Agar). While the washings from sinuses were suspended together with diluted ditiotreitol (DTT) of the same volume, at the concentration of 100 µg/ml, which finally made up the concentration of 50µg/ml. The mixture was allowed to stand at room temperature for 15 minutes. During that time DTT broke the disulfide bonds making the mucus liquid and thus enabling the direct contact between the fungal spores and the medium. Next the mixture was centrifuged at 3000g, the supernatant removed and the residue inoculated on the MA and BHIA media. The plates with the inoculation were then carefully covered with plastic bags and incubated for 3-4 weeks at the temperature of 25°C ± 1°C. The first reading was done after 5 days and then every 7 days roughly. Every grown fungal colony was transferred onto separate mediums for species identification.

The study group was made up of 107 patients (45 women and 62 men). Their age average was 48. In the tested group fungi were detected in 67% of the samples (both in the tissue and nasal lavage fluid). 12 strains of different fungal species (altogether 43) were isolated from the material. Most commonly occurring turned out to be Penicillium (48%) and Aspergillus (15%). The remaining mycological flora constituted Aurenomonium (4%), Alternaria (2%), Cladosporium (11%), Chaetomium (1%), Eurotium (4%), Fusarium (10%), Geotrichum (1%), Verticillium (1%), Rhizopus (1%) and unidentified colonies (8%). Most often isolated species were: Penicillium crustosum, Penicillium citrinum, Aspergillus niger, Cladosporium cladosporioides and Fusarium verticillioides.

The control group (n=12) consisted of patients, at whom no chronic rhinosinusitis was diagnosed; material for study was taken during endoscopic surgery of orbital decompression, DCR-anastomosis of lacrimal sac with middle nasal meatus, or conchotomy. Only the tissue material was analysed, yet fungi were found in none of the samples.

Fungi play an important role in the pathogenesis of rhinosinusitis and although fungal-origin sinusitis is often difficult to diagnose, this factor must be taken into consideration when examining a patient. Proper diagnostics and effective treatment guarantee a therapeutic success.

Acknowledgement
Financial support: Ministry of Science and Higher Education project no NN 305 366039
The present study was aimed at establishing the contamination levels of feed with different fungi, especially *Fusarium* and the impact of zearalenone mycotoxin on the cattle reproduction system.

We collected 10 maize samples from a beef cattle farm in Cluj county and we analyzed them for mycological and mycotoxicological content. The samples originated from the current feed of 140 adult cattle, 80 cows and 60 heifers. The mycological examination has revealed a large load with mycetes of genus *Fusarium*, *Mucor* and *Penicillus* (60-5500 thousand CFU/g). Zearalenone contents showed values between 20 and 2150 µg/kg in maize samples. Abortions occurred in the interval between day 40 and 6 months in 12 cases, ovarian cysts were found in other 7, oestrous haemorrhage in 8 cases and post oestrus haemorrhage in 26 situations, retained placenta followed by pathological puerperium in 18 animals. All reproduction disorders were directly linked to the high zearalenone content in analyzed samples.
Impact on animal and human health – P84

Effect of increasing concentrations of deoxynivalenol (DON) in diet of adult roosters on blood haematology, plasma clinical-chemical parameters and nutrient digestibility

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Among trichothecenes, deoxynivalenol (DON) is a mycotoxin that commonly contaminates cereals worldwide, it occurs frequently in toxicologically levels in grains such as wheat, barley, oats, and maize. Tolerances to DON vary between livestock species and as poultry is regarded rather insensitive to DON it is expected to divert the contaminated cereal batches into poultry feeding. There is a dearth of literature regarding the effects of DON on rooster’s health and nutrient digestibility. The objectives of the present study were, therefore, to investigate the effect of long term feeding of increasing DON concentrations in diets of roosters on haematology, plasma clinical-chemical parameters and nutrient digestibility.

Methods: Twenty four adult roosters of a commercial strain (New Hampshire hybrids) were individually weighed and randomly assigned to individual cages, serving as 8 replicates for each of the three treatment groups. Birds were fed three diets with increasing DON concentrations (1.2, 4.5, 9.4 mg/kg) for 10 months. Feed and water were applied for ad libitum consumption. During the trial, a balance experiment was carried out which lasted for 9-d; roosters were adjusted to a daily feed amount of 90 g; excreta were collected from the plastic trays beneath the cages in the morning and afternoon and kept frozen. Finally excreta were freeze dried and ground and analysed for the nutrients content. At the end of the study, all birds were slaughtered. Blood was collected for haematological evaluation and preparation of plasma. All data were evaluated by ANOVA; means were compared by Tukey-HSD test (p<0.05).

Results: Haematocrit and the differential white blood cell counts were not affected by the dietary treatment. The content of urea was significantly increased in plasma of roosters fed the highest DON concentrations (fig 1). The plasma concentrations of total protein, bilirubin, albumin and cholesterol in addition to the plasma activity of AST, GGT and GLDH were not affected. Moreover, crude fat utilization was progressively increased as the concentrations of DON in the diet increased (fig 2). Utilizations of organic matter, carbohydrates, nitrogen balance and ME were not affected by the dietary treatment.

Conclusion: With the exception of the content of urea in plasma; feeding DON did not affect the blood haematology or plasma clinical-chemistry parameters of the roosters while fat utilization was even improved due to the presence of DON in diet. More studies are needed to clarify these results.
Utilizations of organic matter, carbohydrates, nitrogen balance and ME were not affected by the dietary utilization was progressively increased as the concentrations of DON in the diet increased (fig 2). Addition to the plasma activity of AST, GGT and GLDH were not affected. Moreover, crude fat concentrations (fig 1). The plasma concentrations of total protein, bilirubin, albumin and cholesterol in Results data were evaluated by ANOVA; means were compared by Tukey-HSD test (p<0.05). Excreta were freeze dried and ground and analysed for the nutrients content. At the end of the study, all collected from the plastic trays beneath the cages in the morning and afternoon and kept frozen. Finally was carried out which lasted for 9-d; roosters were adjusted to a daily feed amount of 90 g; excreta were groups. Birds were fed three diets with increasing DON concentrations (1.2, 4.5, 9.4 mg/kg) for 10 Methods: Twenty-four adult roosters of a commercial strain (New Hampshire hybrids) were individually fed ad libitum: The objectives of the pre tolerances to DON vary between livestock species and as poultry is regarded rather insensitive to DON it is expected to divert the contaminated cereal batches into poultry feeding. There is a dearth of literature regarding the effects of DON on rooster’s health and nutrient digestibility. The institute of Animal Nutrition, Friedrich-Loeffler-Institute (FLI), Federal Research Institute for Animal Health, Bundesallee 50, 38116 Braunschweig, Germany. E-mail: mohammad.ebrahem@fli.bund.de

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IMPACT ON ANIMAL AND HUMAN HEALTH – P85

Quantitative assessment of risk associated with dietary intake of mycotoxin ochratoxin A on the adult inhabitants in Shanghai city of P.R. China

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Mycotoxin ochratoxin A (OTA) is considered to be a possible risk factor for adverse effects in humans. It has been found in a wide variety of foods, such as grapes, cereals, beans, dried fruits, as well as their derived products. However, evidence from human studies to evaluate the risk of OTA at current dietary exposures in China is still inadequate. In the present study, quantitative assessment of risk associated with dietary intake of OTA was performed based on consumption habits of the representative adult inhabitants in Shanghai city of P. R. China. Firstly, a total of 400 food samples randomly collected from different locations of Shanghai were analyzed by the previously established isotope dilution LC-MS/MS method. Then, 265 participants of 70 males and 195 females as representative inhabitants were invited to answer the designed questionnaire about the quantity and frequency of foods including four major varieties of grapes, cereals, beans and dried fruits as well as their derived products. Finally, all data were simulated by the point evaluation and model evaluation for the risk assessment of OTA contamination. Results from the point evaluation indicated that mean value of daily intake (DI) of OTA was 1.147 ng/kg body weight/day, which was lower than all the reference standards. However, DI value (8.566 ng/kg body weight/day) in the high percentile (97.5th) was obviously higher than the PTDI (5 ng/kg body weight/day) proposed by Scientific Committee on Food. Among the different groups of foods, OTA in cereals and derived products made the largest contribution to the potential healthy risk. The mean DI value and 97.5th percentile were 1.093 and 7.962 ng/kg body weight/day, respectively, indicating that more than 90% of the risk was due to the contamination of OTA in cereals and derived products. On the other hand, similar results were obtained by the Monte Carlo assessment model. Thus, from the currently available data and analyzed results on the adult inhabitants, regarding OTA contamination issues on food safety administration of Shanghai, there was no significant attention which should be paid on food consumption in Shanghai, besides cereals and derived products with very little possibility as the risk factors.

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Abstracts of posters

IMPACT ON ANIMAL AND HUMAN HEALTH – P86

In vivo evaluation of inorganic and organic anti-mycotoxin additives and their blends in growing broilers

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Sporadic contamination of feed ingredients with mycotoxins has led to routine application of anti-mycotoxin additives in poultry feeding. Most commonly available types are inorganic silicates or clay based additives. Recently however novel organic formulations based on detoxifying enzymes are being used. In this study we evaluated the efficacy of four different anti-mycotoxin additives based on the performance and organ weights parameters in growing broilers. In a 21 day feeding trial, three hundred and thirty 17 day old broilers were randomly allotted to 11 dietary treatments. Each treatment consisted of 3 replicates of 10 birds each. Birds were fed diets supplemented with one or mixture of two anti-mycotoxin additives. Diet 1 was the control (without additive), while diets 2 to 11 contained clay minerals (montmorillonite and silizium dioxide) or enzyme based adsorbent additives. Three commercial clay based adsorbents and one novel enzyme based anti-mycotoxin additive was used for the study. The adsorbents were supplemented as follow: diet 2 was supplemented with clay adsorbent additive A (silicate based) at 1 g/kg, diet 3 with 0.4 g/kg of additive B (enzyme based), diet 4 with 0.2 g/kg of B, diet 5 with 0.2 g/kg of adsorbent C (clay based), diet 6 with 0.4 g/kg of adsorbent C, diets 7 and 8 with 0.5 g/kg and 1 g/kg with adsorbent D (clay based), respectively; diet 9 with 0.2 g/kg of additive B and 0.2 g/kg of C, diet 10 with 0.2 g/kg of B and 0.5 g/kg of adsorbent D and diet 11 with 0.2 g/kg of B and 1 g/kg of adsorbent D. The analyzed mycotoxin contents of the basal diet supplemented with mycotoxin binders were < 50 µg/kg DON, < 30 µg/kg ZEA and 140 ± 40 µg/kg AFlA. The average daily weight gain (ADG), final weight gain and feed conversion ratio (fcr) of birds fed diets with the individual mycotoxin binder or their mix were not significantly influenced (p > 0.05) by the imposed treatments. Nonetheless a trend for diet effect was evident for ADG (p = 0.082) and fcr (p = 0.078). Diet 11 supplemented with 0.2 g/kg of adsorbent B and 1 g/kg of adsorbent D had the highest final body weight (1427 g) and ADG (52 g), while diet 4 supplemented with 0.2 g/kg of additive B had the least fcr (2.14). The heart size (p = 0.08) was not significantly affected, while the liver size (as percentage of live weight) was significantly affected (p < 0.009). The control diet 1 had the largest liver while diet 3 had the least liver size. These results indicated that the additives are not anti-nutritive at the dosages or combinations investigated. However clear improvement in growth performance was not confirmed due to low natural mycotoxin contaminations in the assay diets.
**IMPACT ON ANIMAL AND HUMAN HEALTH – P87**

The ability of different *in vitro* Aflatoxin binding methods to predict the *in vivo* effectiveness of clay binders in broiler diets with high levels of Aflatoxin B₁

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Several *in vitro* models are often used to measure the effectiveness of products to adsorb aflatoxins. However, their value for predicting in vivo efficacy of those products is widely questioned. Therefore five clay products were evaluated with two in vitro methods. The efficacy of two selected clay products was further studied in broiler chickens fed with high levels of aflatoxin.

The two in vitro methods used were the binding efficiency test and the isothermal method. The former measures binding efficiency as adsorption at pH 3 minus desorption at pH 7. The latter incubated the binder at a static pH with several, increasing levels of AFB₁ until an equilibrium state was achieved at each concentration. Four of the five products showed consistent excellent binding efficiency for AFB₁, close to 100%. Isothermal analysis showed a big variety among samples. Only one product could be fitted to the Langmuir model but had a very low Q_{max} of 0.194. The rest of the clays had a better fit for the Freundlich model. Two products, which performed differently in the isothermal method (Products A & B) were further evaluated for their capability to reduce signs of aflatoxicosis in day old broiler chickens. The trial was conducted with two levels of AFB₁ (<5 and 3000ppb) and two levels of each clays (0 and 0.25%). Each treatment consisted of 8 pens with 5 birds per pen. At Day 21, 2 birds per pen were sacrificed to measure relative organ weights.

The broiler chickens showed reduced growth performance but increased relative organ weights of liver, kidneys and gizzard when fed a diet contaminated with 3ppm AFB₁. Product A was significantly better in offsetting the negative impact of AFB₁ on growth and feed intake compared to Product B. Both products were equally effective in reducing the relative organ weights.

In conclusion, the isothermal method showed a better prediction of the *in vivo* effectiveness of the clay products on growth performance compared to the adsorption efficiency method. This study has supported the value of *in vitro* evaluation of aflatoxin binding products.
IMPACT ON ANIMAL AND HUMAN HEALTH – P88

**Serum clinical biochemistry of broilers fed Deoxynivalenol and counteracting agent**

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Deoxynivalenol (DON) is a significant contaminant of broiler feed. It was shown that DON feeding resulted in a reduced vaccinal response and induced stress to broilers. In the present experiment, serum clinical chemical parameters including liver enzymes were investigated in broilers fed DON and detoxifying agent. A total of 40 male broiler chicks (ROSS 308) were reared in battery cages in 4 groups (10 birds / group) according to the following dietary treatments; 1- control group fed basal diet, 2- DON group fed basal diet contaminated with 10 mg/kg diet, 3- DON + Mycofix group fed basal diet contaminated with 10 mg DON/kg diet and supplemented with 2.5 kg of Mycofix Select (MS) (Biomin GmbH, Herzogenburg, Austria) per tonne of diet, 4- Mycofix group fed basal diet supplemented with 2.5 kg of Mycofix Select (MS) (Biomin GmbH, Herzogenburg, Austria) per tonne of diet. At 5 weeks old, blood was collected from all birds and the clinical chemical parameters were determined in the serum. Glucose, aspartate transaminase (AST), alanine transaminase (ALT), cholesterol, uric acid, total protein, albumin and globulins, gamma glutamyl transpeptidase (GGT), triglycerides, lactic acid dehydrogenase (LDH), and creatinine kinase were determined in serum by an automatic clinical chemistry analyzer (COBAS 501C, Roche, Vienna, Austria). The results showed that DON increased serum cholesterol and decreased serum ALT (Table 1). GGT (gamma glutamyl transpeptidase) was numerically increased due to DON feeding. However, LDH (lactic acid dehydrogenase), creatinine kinase and AST (aspartate transaminase) remained unaffected by dietary DON. Moreover, DON significantly increased serum triglycerides and addition of Mycofix decreased triglycerides compared with controls. Mycofix Select slightly decreased GGT. However, MS can not change the adverse effects of DON on serum cholesterol and GGT. Glucose was reduced and uric acid was increased by MS. Total protein, albumin and globulin were increased by MS.

Table 1. Clinical chemical serum parameters of broilers fed Deoxynivalenol and detoxifying agent

<table>
<thead>
<tr>
<th>Item</th>
<th>Control group</th>
<th>DON group</th>
<th>DON + Mycofix</th>
<th>Mycofix Group</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mg/dl)</td>
<td>260±8</td>
<td>257±5</td>
<td>245±4</td>
<td>242±4</td>
<td>0.090</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>274±16</td>
<td>262±24</td>
<td>243±9</td>
<td>265±20</td>
<td>0.685</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>4.6±0.5</td>
<td>4.2±0.5</td>
<td>4.6±0.3</td>
<td>4.9±0.4</td>
<td>0.005</td>
</tr>
<tr>
<td>Cholesterol (mg/dl)</td>
<td>123±5</td>
<td>144±6</td>
<td>158±7</td>
<td>143±6</td>
<td>0.002</td>
</tr>
<tr>
<td>Uric acid (mg/dl)</td>
<td>0.85±0.23</td>
<td>0.81±0.17</td>
<td>0.95±0.23</td>
<td>1.36±0.29</td>
<td>0.081</td>
</tr>
<tr>
<td>Total protein (g/dl)</td>
<td>2.80±0.05</td>
<td>2.91±0.07</td>
<td>3.06±0.09</td>
<td>3.06±0.13</td>
<td>0.073</td>
</tr>
<tr>
<td>Albumin (g/dl)</td>
<td>1.24±0.03</td>
<td>1.29±0.03</td>
<td>1.39±0.03</td>
<td>1.36±0.05</td>
<td>0.038</td>
</tr>
<tr>
<td>Globulin (g/dl)</td>
<td>1.56±0.04</td>
<td>1.62±0.05</td>
<td>1.67±0.07</td>
<td>1.69±0.08</td>
<td>0.102</td>
</tr>
<tr>
<td>Albumin/Globulin ratio</td>
<td>0.79±0.03</td>
<td>0.81±0.04</td>
<td>0.84±0.02</td>
<td>0.81±0.03</td>
<td>0.746</td>
</tr>
<tr>
<td>GGT (U/L)</td>
<td>17.78±1.47</td>
<td>19.30±1.69</td>
<td>21±2</td>
<td>18.70±1.56</td>
<td>0.599</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>39.55±4.79</td>
<td>65.23±7.50</td>
<td>58.80±9.79</td>
<td>47.20±4.29</td>
<td>0.074</td>
</tr>
<tr>
<td>LDH (U/L)</td>
<td>103±72</td>
<td>941±114</td>
<td>754±129</td>
<td>103±283</td>
<td>0.586</td>
</tr>
<tr>
<td>Creatinine kinase (U/L)</td>
<td>12342±1888</td>
<td>11542±2769</td>
<td>8315±1302</td>
<td>13262±3926</td>
<td>0.590</td>
</tr>
</tbody>
</table>

a,b Means within the same row with different superscripts are significantly different (ANOVA followed by Duncan test; n = 10/treatment).

From this experiment, it can be concluded that, the decreased ALT in a combination with increased cholesterol after DON feeding suggests liver problem and/or kidney affection. DON had adverse effects on the liver enzymes and lipid metabolism. Such effects of DON indicated that it could affect the broilers health due to its chronic toxicity. In addition, Mycofix Select might partially counteract these effects.
IMPACT ON ANIMAL AND HUMAN HEALTH – P89
Cyclopiazonic acid: toxic effects in broilers and the role of oxidative and nitrosative stress

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The excessive generation of Reactive oxygen species (ROS) and nitrogen species (RNS) has been implicated in the pathogenesis of numerous diseases. Cyclopiazonic acid (CPA) is an indol-tetramic acid mycotoxin, which is produced by several fungal of the genera Penicillium and Aspergillus. The occurrence of CPA has been reported in human foods such as peanuts, wheat, soybean, and cheese products. There is also evidence indicating the occurrence of CPA in animal feeds including oats and other grains, and subsequently in poultry diets. The aim of this study is to identify the role of reactive oxygen/nitrogen species (ROS/RNS) in pathological changes in the liver and kidneys of broilers following a sub-acute exposure to CPA (1, 2). Ten-day-old male broiler chicks (Ross 308, n=40) were assigned into control and test groups, which received normal saline and 10, 25, and 50 mg/kg CPA, respectively, for 28 days. Body weight gain, serum level of alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), uric acid, creatinine, and blood urea nitrogen (BUN) were measured after 2 and 4 weeks exposure. Moreover, the total thiol molecules (TTM), malondialdehyde (MDA), nitric oxide (NO) contents and also mRNA level of inducible nitric oxide synthase (iNOS) in liver and kidneys were assessed. To confirm the biochemical changes, histopathological examinations were conducted on either organ. The MDA and NO content of the liver and kidneys showed a significant (P<0.05) increase in a dose-dependent manner. In contrast, the TTM levels in the liver and kidneys were significantly (P<0.05) attenuated. The mRNA level of iNOS in birds of the CPA test groups showed a reverse relationship with the NO increase. These alterations of ROS/RNS level were associated with an increase in ALP and GGT level in serum in comparison to the control group. CPA exposure also resulted in uric acid, creatinine and BUN enhancement in broilers.

In conclusion, these findings suggest that exposure to CPA results in hepatic and renal disorders via oxidative and nitrosative stress, which were reflected as biochemical changes and pathological injuries in either organ. The elevated MDA and NO content and equally the attenuation of the antioxidant capacity due to CPA exposure may result in inflammatory response in target tissues, making the animals susceptible to pathological alterations.

References
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Efficacy of a hydrated sodium calcium aluminosilicate to reduce aflatoxin residues in liver and kidney of broiler chicks fed aflatoxin B₁

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Aflatoxin B₁ (AFB₁) is a potent mutagen and hepatocarcinogen produced by some species of Aspergillus and is found in a wide range of food and agricultural products. Poultry fed AFB₁ contaminated rations under experimental conditions had AFB₁ residues in tissues such as liver and kidney, which are the main organs involved in the detoxification of AFB₁ (Leeson et al., 1995). The addition of hydrated sodium calcium aluminosilicate (HSCAS) to AFB₁ contaminated diets has been shown to greatly reduce the bioavailability of AFB₁ in the gastrointestinal tract of poultry (Kubena et al., 1990). The objectives of this study were to determine the in vitro binding capacity (Qₘₐₓ) of an HSCAS for AFB₁ and the efficacy of an HSCAS to reduce residual concentrations of AFB₁ metabolites in the liver and kidney of broilers fed AFB₁. Different concentrations of the HSCAS (0.05, 0.25, 0.5, 1, 10 and 100 mg/10 mL) were added to tubes containing phosphate buffer (0.1 M) adjusted to pH 3.0 containing 2 mg/L of AFB₁. Samples were placed on a rotator shaker for 30 minutes at room temperature, centrifuged at 13,000 rpm for 5 minutes and 1.0 mL of the supernatant was analyzed by high performance liquid chromatography (HPLC). One hundred day-old male broilers (Ross 708) were maintained in chick batteries and allowed libitum access to feed and water. A completely randomized design was used with 5 replicate pens of 5 chicks assigned to each of 4 dietary treatments from hatch to 21 days. Dietary treatments included: A) basal diet (BD), with no HSCAS or AFB₁; B) BD supplemented with 0.5% HSCAS only; C) BD supplemented with 2.5 mg AFB₁/kg of feed; and D) BD supplemented with 2.5 mg AFB₁/kg of feed and 0.5% HSCAS. On day 21, 5 chicks from each treatment were anesthetized with carbon dioxide and killed by cervical dislocation. Samples of liver and kidney were collected for analysis of AFB₁ and aflatoxicol residues by using immunoaffinity columns (Aflatest, Vicam) for clean-up and determination by HPLC according to Scott (1990). The percentage of AFB₁ bound in vitro for each concentration of adsorbent (0.05, 0.25, 0.5, 1, 10 and 100 mg/10 mL) was, 8.8, 40.1, 75.4, 81.8, 91.1 and 100%, respectively. The maximum amount (Qₘₐₓ) of AFB₁ adsorbed by the HSCAS was 48.02 mg/g. Concentrations of AFB₁ and aflatoxicol were lower (P < 0.05) in livers of birds fed AFB₁ plus HSCAS as compared to birds fed AFB₁ alone. Concentrations of AFB₁ were also lower (P < 0.05) in kidneys of birds fed AFB₁ plus HSCAS compared to those fed AFB₁ only. HSCAS was shown to adsorb AFB₁, having a high sorption stability, thereby limiting AFB₁ bioavailability, reducing aflatoxin residues and protecting broilers from aflatoxicosis.

References
Development of an animal model for assessing the toxicity of fumonisin derivates

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Fumonisins are frequent contaminants of cereal based foods and exhibit cancerogenic, hepatotoxic, nephrotoxic as well as immunosuppressive effects in mammals. Their toxicity is based on disruption of the sphingolipid metabolism. The ratios of the sphingoid bases sphinganine (Sa) and sphingosine (So) and of their respective phosphates in plasma, urine or tissues are used as biomarkers for fumonisin exposure. Besides fumonisin B1 (FB1), there exist numerous fumonisin metabolites, such as hydrolyzed fumonisin B1 (HFB1), N-acetyl-HFB1 or N-(1-deoxy-D-fructos-l-yl)-FB1. They are either occurring naturally in cereal crops or formed during food processing. The toxicological relevance of these metabolites is not yet fully known.

The aim of our study was to compare the toxicities of HFB1 and FB1 based on the Sa/So ratio in different biological matrices of rats. Data obtained in this experiment will facilitate the establishment of an animal model for the toxicity assessment of different fumonisin metabolites.

20 male Sprague Dawley rats were divided into 5 groups (n=4): negative control, FB1 low (13.9 µmol FB1/kg feed), FB1 high (69.3 µmol FB1/kg), HFB1 low (13.9 µmol HFB1/kg) and HFB1 high (69.3 µmol HFB1/kg). The animals received the diets and water ad libitum for 4 weeks. On days 7, 14, 21 and 28 the rats were housed individually in metabolic cages for 24 hours to collect urine and feces. On day 28, animals were anesthetized, euthanized and serum and tissue samples (kidney, liver, lung) were taken. After sample preparation, analyses of sphingoid bases were carried out by LC-MS/MS.

On day 28, urinary Sa/So ratios of 0.03 ± 0.03, 3.65 ± 0.84, 7.14 ± 1.06, 0.14 ± 0.10 and 0.05 ± 0.03 were determined for the negative control, FB1 low, FB1 high, HFB1 low and HFB1 high. The differences between both FB1- and HFB1-groups as well as between the FB1-groups and the negative control were statistically significant. These differences were also displayed in the results of kidney sample analysis, whereas no elevation of the Sa/So ratio was detected in any of the other investigated matrices (serum, liver, lung).

Results confirm that HFB1 does not disrupt sphingolipid metabolism in rats, as previously shown for mice by Howard et al. (2002). In our experiment, assessment of HFB1 toxicity included for the first time the determination of urinary Sa/So levels. Due to the non invasive sampling procedure and the significance of the obtained results, the analysis of urine proved best suited for our purposes and will be implemented in future toxicity assessment of other fumonisin metabolites.

References
Correlating specific and unspecific parameters for efficacy testing of mycotoxin detoxifying agents using deoxynivalenol in turkey poults

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Mycotoxin binders are commonly added in animal feed to counteract the negative effects of mycotoxins on production animals (Kolosova and Stroka, 2011). It is obvious that these products should be tested for their mycotoxin adsorbing ability in the gut. Discussion is ongoing, however, by which method these products should be evaluated in vivo. Mycotoxin binders are generally evaluated based on unspecific parameters such as performance data, histological changes and/or alterations in immune responses. These criteria are unspecific and do not show a direct correlation with the adsorption capacity in the intestinal tract. Other, direct end-points have recently been proposed by the European Food Safety Authority (EFSA) (EFSA, 2010). These are based on specific, toxicokinetic parameters. The most relevant end-point for deoxynivalenol (DON), for example, is DON and the main metabolite de-epoxydeoxynivalenol (DOM-1). The goal of this study was to evaluate a possible correlation between both sets of parameters using DON and a glucomannan (GMA) mycotoxin binder (Mycosorb®, Alltech Inc., KY, USA) in turkey poults. Two hundred forty one-day-old turkeys were randomly allocated to one of four diets: (1) control (minimally contaminated) (2) control + 0.2 % GMA (3) naturally contaminated (4-6 mg DON/kg feed) (4) naturally contaminated + 0.2 % GMA. At the end of starter (3 w), grower (6 w), developer (9 w) and finisher (12 w) phases, plasma concentrations of DON and DOM-1, body weight gain, feed intake, feed conversion rate and plasma biochemistry were evaluated. At the end of the starter phase, duodenal sections were excised from 4 birds/pen (12/diet) for histological (morphometry) and immunohistochemistry analysis (CD8+ T-lymphocyte counts). The results of performance parameters showed an increased body weight (gain) in the starter phase of the animals fed the DON contaminated diet compared to controls. The plasma biochemistry profile showed some statistically significant effects of diet on several minerals and enzymes. This was, however, inconsistent over the twelve week period. Histological morphometry analysis showed significantly lower villus height and apparent villus surface area in birds fed the contaminated diet compared to controls but this was not seen in birds fed the contaminated+GMA diet. An increased level of CD8+ T-lymphocytes was observed in birds fed the contaminated diet as well as contaminated+GMA diet compared to controls. No significant differences in DON nor DOM-1 plasma concentrations were observed comparing birds fed the contaminated and contaminated+GMA diets but both were elevated compared to controls. It can be concluded that GMA could alter some negative effects of DON on unspecific parameters, but was not able to lower the oral bioavailability of DON or DOM-1.

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References
Deoxynivalenol impairs proximal intestinal barrier in broiler chickens, but addition of an adsorbing agent shifts the effects to more distal

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Broiler chickens are reported to be rather resistant to deoxynivalenol (DON) and thus, clinical signs are rarely seen. However, effects of deoxynivalenol in the feed at the molecular level in the intestines are less frequently studied. In this study, we investigated the effects of three weeks of feeding 7.5 mg deoxynivalenol/kg feed on the gut wall morphology, intestinal barrier function and inflammation in broiler chickens. In addition, oxidative stress was evaluated and the effect of a clay-based mycotoxin adsorbing agent on these different parameters was also studied. Our results show that feeding deoxynivalenol affects the gut wall morphology both in duodenum and jejunum of broiler chickens. A qRT-PCR analysis revealed that deoxynivalenol acts in a very specific way on the intestinal barrier, since only an up-regulation in mRNA expression of the tight junction protein claudin 5 in jejunum was observed, while no effects were seen on claudin 1, zona occludens 1 and 2. Remarkably, addition of an adsorbing agent to DON contaminated feed resulted in an up-regulation of all investigated genes coding for the intestinal barrier in the ileum. Up-regulation of Toll-like receptor 4 and two markers of oxidative stress (heme-oxygenase and xanthine oxidoreductase) was mainly seen in the jejunum and to a lesser extent in the ileum in response to deoxynivalenol, while in combination with an adsorbing agent, again the main effect was seen in the ileum. These results suggest that an adsorbing agent may lead to prolonged presence of deoxynivalenol in the small intestines, with a possible release again of the toxin in the more distal parts.

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Study on the mechanism of sperm damage in balb/C mice exposed to Deoxynivalenol

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In this study the effect of deoxynivalenol (DON) exposure on sperm and testis damage were assessed in BALB/c mouse. Male mice were first divided into control group (n=15) and treatment group (2.4 mg/kg DON, n=15), and then exposed to DON daily for 30 days via intragastric administration. The results showed as follows: compared with control, the ration of seminal vesicle to body, and sperm survival and motility were significantly decreased ($P<0.05$ or $P<0.01$), respectively. Furthermore, acrosome absent, the mitochondrial sheath shedded in midpiece, tail curved, and double head or double tail of spermatozoon were observed in DON treatment group. In addition, the abnormal sperm structure under transmission electron microscope was found with impaired plasma membrane, irregularly arranged mitochondrion, vacuoles presence in mitochondrion, and thin mitochondrial sheath. In testis, seminiferous epithelium in contorted seminiferous tubules was much thinker, spermatid were irregularly organized, and the number of sperm was also less present. Moreover, the concentration of reactive oxygen species (ROS) and glutathione (GSH) in testis were significantly increased compared with control ($P<0.05$). Then, the expression of JNK, c-Jun, p-JNK, p-c-Jun and mRNA levels of Bim, Caspase-8, Caspase-3, Caspase-9 were much higher than control with significance ($P<0.05$), respectively. It is concluded that the mechanism of sperm damage caused by DON is that DON enhances the testis ROS and GSH content, leads to the oxidative stress and actives the JNK/c-Jun cell signaling, then triggers caspase-3 mitochondrion apoptosis pathway and induces germ cells apoptosis. As concluded, testis damage may contribute to the spermatogenesis deficiency and abnormality rate increase, and indicate low fertility.

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Characterization of *in vitro* intestinal absorption of veterinary drugs and coccidiostats in the presence of mycotoxin detoxifiers using a porcine intestinal epithelial cell line

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Adding mycotoxin binders and modifiers to feed is a widespread strategy to reduce the effects of mycotoxins on animals. These products are often registered as technological additives and are considered safe for the animal. However, only few studies are available that investigated the interaction of these additives with other substances added to the feed such as coccidiostats and veterinary drugs (Goossens et. al, 2012; Osselaere et. al, 2012). A large scale screening method is also not yet available. This study aims to develop and apply an *in vitro* model to characterize the absorptive behavior of xenobiotics in the presence of mycotoxin detoxifying agents and of mycotoxins. Therefore, an intestinal porcine intestinal epithelial cell line (IPEC-J2) will first be cultured on a Transwell® insert system (Devreese et. al. 2013). Following, a mixture of veterinary active substances, together with a mycotoxin detoxifier and a mycotoxin will be applied on the Transwell®. The influence of twelve different detoxifiers on the cellular passage of twelve orally applied veterinary drugs in poultry and pigs such as beta-lactams, aminoglycosides, tetracyclines, macrolides, lincosamides, pleuromutilins, sulfonamides, trimethoprim, fluoroquinolones and sodium salicylate will be studied. Furthermore, nine coccidiostats including ionophoric coccidiostats, nicarbazine, halofuginone, robenidine and diclazuril, also frequently used in poultry and pigs, will be assessed. The mycotoxins which will be included in this study are deoxynivalenol and aflatoxin B1. Control groups without mycotoxins and/or detoxifiers will also be included. The passage of the drugs and coccidiostats will be monitored using in-house developed LC-MS² methods and the permeability coefficients will be compared with the control groups. To exclude drug-drug interactions, the passage characteristics of the mixture of drugs and coccidiostats will be compared with the absorptive behavior of the single drug or coccidiostat. Results will be presented at the workshop.

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References


Evaluation of 2 commercial products to counteract the effects of T-2 toxin on lesions and on performance in broilers

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The present study was undertaken to evaluate the efficacy of 2 commercial products in counteracting the negative effects of T-2 toxin in broilers with respect to lesion formation and performance. One hundred twenty 1-day-old male Ross 308 broilers were randomly divided into 4 dietary treatments 1) control (no T-2), 2) 1.5 ppm T-2, 3) 1.5 ppm T-2 + 2.0 kg/T Product A and 4) 1.5 ppm T-2 + 1.0 kg/T Product B. When no commercial product was included, 1.5 ppm T-2 toxin decreased body weight (BW) and increased feed conversion (FC). When Product A was added to the contaminated feed, the birds were protected against the negative effect of T-2 on BW and FC. Addition of Product B, resulted in even worse performance. T-2 caused more birds with oral lesions compared to the control group. Supplementation of Product A or Product B reduced the number of birds with oral lesions. Unlike Product A the treatment group with Product B showed more birds with more severe lesions. The relative liver weight (RLW) slightly increased by T-2, but decreased by addition of Product A, while by addition of Product B the RLW increased even more. This study showed that T-2 could adversely affect performance and health of broilers. The addition of 2.0 kg/T Product A could completely overcome the negative effects on performance and reduced the number of lesions by 50 %.
Dose response study based in vitro selection of an adsorbent capable to alleviate the negative in vivo effects of zearalenone in female weaned piglets

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In vitro analysis of mycotoxin adsorption is a powerful screening tool to evaluate the potential of mycotoxin detoxifying adsorbents. The single concentration method measures adsorption of a fixed mycotoxin concentration that is reacted with a fixed concentration of adsorbent in an aqueous solution. It reflects the capacity of an adsorbent at one specific ratio of adsorbent to mycotoxin and is the most widely used in vitro method. Dose response studies are a more elaborated test system where mycotoxin adsorption is evaluated in function of varying adsorbent to mycotoxin ratios. The adsorbent concentrations are varied at a fixed mycotoxin concentration. From dose response curves, the C_{50} value, the binder to mycotoxin ratio for which 50% of the mycotoxin is bound, can be calculated. The C_{50} value is closely related to the BC_{50} value, the binder concentration necessary to ensure binding of 50% of the mycotoxin and the related distribution coefficient K_d, the ratio of the concentration of toxin adsorbed C_{ads} to the concentration of toxin in solution C_{aq}. Both K_d and BC_{50} can be used to quantitatively compare experimental results obtained under widely variable conditions. The in vitro zearalenone (ZEA) adsorption of different adsorbents was evaluated with single concentration and dose response adsorption studies. From both studies, BC_{50} and K_d were determined. The more elaborated dose response studies offered more differentiation potential than single concentration studies.

Based on the in vitro results, the adsorbent with the greatest potential was evaluated in an in vivo trial for six weeks. Sixty female weaned piglets were randomly assigned to five different treatments with three replicates within each treatment and four piglets per replicate. The negative control treatment was fed a low ZEA contaminated diet (0.065 ppm) while the four other treatments were fed a diet with a ZEA contamination of about 1 ppm and an adsorbent dosage of 0, 1, 2 and 3 kg/T. In vivo ZEA adsorption, beta-estradiol serum concentration and vulva size were recorded for all treatments. Histological analyses of follicular and acinar cells were performed on uterus and ovary samples. Feeding female piglets with 1 ppm ZEA exerted hyperestrogenism effects such as vulva swelling and depressed serum beta-estradiol levels. Follicular and acinar cells were reduced after ZEA exposure. The adsorbent was able to alleviate the ZEA induced effects to the same level as in the negative control treatment. These observations were supported by an increased in vivo ZEA adsorption of the adsorbent in a dose dependent manner.

References
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Effects of microorganism-mycotoxins co-contamination in the pig intestinal innate immune response: an in vitro study

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The innate immune system has the basic function of identifying and eradicating microbial invaders and alerting the adaptive immune system to their presence. On the other hand, animal feed is commonly subject to more than one contaminant from different sources (co-contamination). One of the most often reported co-contamination in pig farm includes microorganisms (Escherichia coli, Salmonella etc) and mycotoxins (aflatoxins, deoxynivalenol, zearalenone etc), which are the most frequent natural contaminants of cereals. Many of the previous studies analysed the effect produced by these contaminants as individual rather than the interaction between them. The present study investigated the effect on intestinal immune response of the co-contamination between Escherichia coli as one of the microorganisms responsible for post weaning diarrhoea in pigs and frequent Fusarium mycotoxin zearalenone (ZEA) to which the pig is very sensitive. The effect of the selected co-contaminants (Escherichia coli and ZEA) was evaluated by analysing the expressions of TLRs and cytokines in a porcine epithelial cell line derived from jejunum, IPEC-1. Cells were cultured in the presence or absence of 25 µM of ZEA, and of Escherichia coli. Total RNA was extracted at 1h, 2h and 24h after treatment. Expressions of TLR-1, -2, -3, -4, -6, -8, -9 and -10 and of cytokines (TNF-alpha, IL-8, IL-10) were evaluated by Real Time PCR and ELISA. In Real Time PCR, as endogenous reference were used beta-Actin and Cyclophilin A. Our results showed a time-dependent variability in both TLRs and cytokines gene expression as response to Escherichia coli- ZEA co-contamination. For example, treatments with ZEA and Escherichia coli alone decrease gene expression for TLR-2, -3, -4 and -6 after 1 hour, while combined treatment with ZEA and Escherichia coli lead to an increase of TLR-1 and -6 mRNAs. For the other TLRs, co-contamination has more pronounced effect in decreasing gene expressions. After 2 hours of treatment, ZEA and Escherichia coli alone decreased gene expression for TLR-1, -4 and -6, ZEA induce also an increase in TLR-2, while Escherichia coli treatment was associated with an increase in TLR-3 and -8 mRNAs. Co-contamination leads to a decrease in gene expression for all TLRs after 2 hours of treatment. Regarding cytokines, ZEA and Escherichia coli action at individually level is different, but co-contamination lead to a slightly decrease of IL-8 secretion and to an increase of concentration of secreted TNF-alpha and IL-10.

In conclusion, our study contributes to a better understanding of the interaction between contaminants of feed and food, in the gut as a barrier encountered by exogenous food compounds, and also as an active component of the immune system. Our results represents additional data that can be taken into account in evaluating the effects of microorganisms-mycotoxins co-contamination in the intestinal immune response as well as the consequence in terms of susceptibility to enteric disease.
Due to their polar properties the oral bioavailability of fumonisins is generally low. For fumonisin B1 (FB1) in laying hens it is estimated around 1-2%. Fumonisins frequently co-occur in animal feed with the trichotheccene deoxynivalenol (DON). The objective of this study was to evaluate whether a chronic exposure to DON affects the oral bioavailability of fumonisins in broiler chickens.

Twelve one-day-old Ross 308 broilers were randomly divided into two groups of 6 birds. Throughout the three week experiment one group received a mycotoxin blank diet, while the other group received a diet experimentally contaminated with DON. Concentrations in the feed were determined using a validated LC-MS/MS method (Monbaliu et al., 2010). The contaminated feed contained DON at respectively 3113 ± 900 µg/kg for the starter feed, 2884 ± 800 µg/kg for the grower feed and 3351 ± 1000 µg/kg for the finisher feed. At day 21 all birds received an intra-crop bolus of fumonisins (2.5 mg/kg BW; consisting of 1.91 mg FB1/kg BW and 0.59 mg FB2/kg BW). This concentration was extrapolated from the European guideline level (20,000 µg FB1+FB2/kg feed) and the expected chicken feed intake. Subsequently, blood was withdrawn at several time points after fumonisin administration. Plasma concentrations of FB1, DON and DOM-1 were determined using a validated LC-MS/MS method (Devreese et al., 2012).

No DON nor FB1 and FB2 could be detected in the blank diet above the limit of quantification (LOQ). No differences could be detected in the toxicokinetics of fumonisins between chickens receiving blank and DON contaminated diet (P>0.05). Maximal plasma concentration for FB1 was respectively 37.3 ± 30.0 and 40.3 ± 28.8 ng/ml; time to Cmax was respectively 15.0 ± 5.8 and 15.0 ± 5.5 minutes and area under the plasma concentration-time curve (AUC0-240min) respectively 1717.8 ± 1102.1 and 1818.3 ± 489.3 ng.min/ml. No DON or DOM-1 plasma levels above LOQ were observed in both groups.

In conclusion, chronic exposure to DON does not influence the oral resorption of fumonisins in broilers.

References
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**Effects of T-2 Toxin on the Interaction between Chicken Macrophages and *Aspergillus fumigatus* Conidia**

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*Aspergillus fumigatus* is the primary pathogen of avian aspergillosis. The airborne conidia of this fungus are easily inhaled by a bird and then infect the respiratory system. The respiratory macrophages are responsible for phagocytosing and killing the invading fungal conidia [1]. T-2 toxin (T-2) is a trichothecene mycotoxin with immunomodulatory effect. It is usually produced by *Fusarium* spp. in poorly stored agricultural products such as barley, oat, maize, wheat and rye [2]. To evaluate how T-2 possibly affects the interaction between chicken macrophages and *A. fumigatus* conidia, the macrophages were first treated with T-2 at different concentrations, and then their viability, antifungal activity, and cytokine expression in response to *A. fumigatus* conidial infection were assessed. T-2 at concentrations higher than 1 ng/ml significantly decreased the viability of macrophages. At 1 h p.i., 59% and 30% of the untreated macrophages associated and phagocytosed conidia respectively, while 26% and 5% of the macrophages treated with 5 ng/ml T-2 associated and phagocytosed conidia respectively. The germination rate of conidia associated with untreated macrophages at 7 h p.i. was 24%, in contrast to 75% in macrophages treated with 5 ng/ml T-2. Expression of cytokines IL-1β, CXCL1, CXCL2 and IL-12β in macrophages was significantly upregulated by *A. fumigatus* conidia from 6, 4, 4 and 12 h p.i., respectively, and that of TGF-β4 was significantly downregulated from 12 h p.i. T-2 treatments at 1 to 5 ng/ml further upregulated the expression of IL-1β, IL-6, CCL2, CXCL1, CXCL2, IL-12β and IL-18 (at 1 and 2 ng/ml) in *A. fumigatus*-infected macrophages, but downregulated that of TGF-β4 (at 5 ng/ml) 6 h p.i. In summary, T-2 impaired the antifungal activity of chicken macrophages against *A. fumigatus* conidia, but might promote pro-inflammatory responses caused by conidial infection.

**References**


2. European Food Safety Authority, 2011. Scientific Opinion on the risks for animal and public health related to the presence of T-2 and HT-2 toxin in food and feed. EFSA Journal 9, 2481-2668.
Mycotoxin contamination in commercial parakeet feeds

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Mycotoxins are toxic secondary metabolites of fungi. Animal feeds can be easily infected by fungal species during production and storage, resulting in mycotoxin contamination. Mycotoxins in 10 commercial parakeet feeds (5 seed mixed feeds and 5 pelleted feeds) were analyzed by LC-MS/MS. Among all the 21 analyzed mycotoxins, zearalenone was the most prevalently detected. Feed 1 was the least contaminated pelleted feed. Mycotoxins detected in Feed 2 (pellet) were mainly deoxynivalenol, fumonisin-B1, and zearalenone, and that in Feed 3 (pellet) was fumonisin-B1. Based on these analytical results, Feed 1, Feed 2 and Feed 3 were used in the subsequent in-vivo trial with 15 cockatiels, which were divided into 3 groups (Groups 1, 2 and 3), with 5 birds in each group, fed Feed 1, Feed 2 and Feed 3 respectively for 21 days. Group 1 served as the control. Body weight and weight of liver, gizzard, spleen, heart, kidney, lung, proventriculus, pancreas and bursa of Fabricius of each cockatiel were measured. Mean values of body weight gain or organ weights in Group 2 and Group 3 were not significantly different from those of Group 1, but increase of organ weights of spleen and liver was observed in one bird of Group 2, and in kidney and proventriculus of one bird in Group 3. Severe diarrhea was also observed in the same bird of Group 3. Histological examination revealed lesions in liver, kidney, and spleen in Group 2, and in kidney and proventriculus in Group 3. In conclusion, the existence of mycotoxins is common in parakeet feeds, and this is a risk for the health of psittacines.

References
IMPACT ON ANIMAL AND HUMAN HEALTH – P102

Study of in vivo efficacy of cholestyramine as mycotoxin binder

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The aim of this study was to investigate the in vivo efficacy of cholestyramine as a putative mycotoxin binding agent towards zearalenone (ZEN) and deoxynivalenol (DON). 24 rats were divided into 4 groups, with each 6 animals. In a first group (group I), animals received feed contaminated with ZEN and DON; in a second group (group II), animals received the binder cholestyramine included in non-contaminated feed. The animals from group III received both ZEN/DON and cholestyramine via the feed, and finally, the animals from group IV received a control diet. The contaminated feed was in-house produced by inoculation of a Fusarium graminearum strain on wheat grains. The feed contaminated with mycotoxins (group I and III) contained 2058 ± 103 µg/kg ZEN and 4355 ± 177 µg/kg DON, the feed used as control feed (group IV) or to include the binder cholestyramine (group II) contained 80 ± 4 µg/kg DON. The binder cholestyramine was included in the feed at a level of 1% (group II and III). After 1 week, the animals were sacrificed and blood was sampled for analysis of ZEN, DON and their metabolites by UPLC-MS/MS.

No mycotoxins were detected in any of the serum samples from group II or group IV. In contrast, DON, DOM-1, DOM-3-GU and ZEN-14-GU were detected in the serum samples from group I (ZEN/DON) and group III (ZEN/DON + binder). DOM-1 and DOM-3-GU were only detected in traces in some animals, whereas DON was measured in almost all animals, with mean concentration 2.41 ± 0.93 µg/L. DON in group I and 2.14 ± 0.87 µg/L DON in group III. ZEN-14-GU was detected in all the animals from group I and group III, the mean area of the chromatograms in group I is 808.9 ± 751.3 for ZEN-14-GU and in group III 512.1 ± 133.8.

So, the main mycotoxins detected in the serum of the treated animals were DON and ZEN-14-GU. No differences were found between group I (ZEN/DON) and group III (ZEN/DON + binder). At 1% the binder cholestyramine did not have an effect on the level of ZEN or DON in the serum of rats.
Fumigaclavine A (FuA) is an indole alkaloid belonging to the large group of ergolines (clavine alkaloids). Production of clavine alkaloids has been reported for species belonging to several fungal genera, including Aspergillus (e.g. A. fumigatus), Penicillium (e.g. P. roqueforti) and Claviceps (e.g. C. purpurea). Aspergillosis is one of the most important respiratory diseases in birds, in particular in parrots, waterfowl and birds of prey. Except for gliotoxin nothing is known concerning mycotoxin production in infected birds in vivo. The applicability of a competitive indirect enzyme immunoassay (EIA) for FuA has been demonstrated previously. With a detection limit of FuA EIA in respiratory tissue samples of 1.5 ng/g and recoveries in a concentration range of 5 - 100 ng/g of 84 - 109%, the test was considered to be sufficiently sensitive. Here we report further studies aiming at FuA determination in avian respiratory tissue and in blood serum of falcons with clinical aspergillosis. Our hypothesis was that FuA blood levels could possibly provide a convenient marker for avian aspergillosis in vivo, without the need to perform endoscopic examinations.

FuA was directly analysed in respiratory tissue of birds died with aspergillosis. The test was also evaluated for the FuA determination in diluted avian blood serum samples previously treated with glucuronidase/sulfatase. The detection limit for FuA in blood was found to be 0.7 ng/ml, recoveries at a FuA level of 20 ng/ml were at 65.5%. No FuA could be detected in negative control blood samples and in 15 blood samples of falcons infected with aspergillosis. Since relatively high FuA levels exceeding 10 ng/g were found in avian respiratory tract samples colonized by Aspergillus spp., this indicates that either FuA does not migrate into the bloodstream at significant levels, or that FuA is metabolized yielding a modified chemical structure, which is non cleavable by glucuronidase/sulfatase and non-reactive in the EIA. In conclusion, FuA determination in avian blood does not provide a means for early detection of aspergillosis.
Is mycotoxin contaminated-pet food responsible of reduced fertility and stillbirth?

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As cereal grain and nuts are often used as ingredients in industrial pet food, companion animals such cat and dog are exposed to mycotoxins. Some owners and breeder of cats and dogs observed a decrease of fertility and stillbirth. As all other causes (virus…) have been ruled out, it has been hypothesised that feed contaminants should be involved especially when replacement of this feed by another without cereals avoid the problems. The aim of this study was to analyse the occurrence of mycotoxins in feed which has induced problems. In parallel mycotoxins were analysed in tissues (blood of mother and in liver, kidney, intestine, brain of litters). DNA adducts reflecting genotoxicity were analysed by 32P-post labelling. Some cats gave birth to monstrous litters with intestines outside the abdomen, cleft lip, without fur, wasted legs (figure 1).

All the pet food leading to reproductive problems are contaminated by several mycotoxins: OTA, AFB1 (+ other metabolites AFB2, AFG1, AFG2), CIT, ZEA, FB1, DON. The pet food without cereal contains much less mycotoxins and enabling normal birth. Large amount of OTA, ZEA, AF and their metabolites and FBs were found in blood, kidney, liver, intestine, brain and placenta from monstrous cat (Table 1). Specific DNA adducts (related to OTA and/or ZEA) were detected in the tissue of little cat. Although the amounts of individual mycotoxins seem to be low, the simultaneous occurrence of these mycotoxins in pet food due to a synergistic effect seems to be responsible of the reduce fertility and still birth.

### Table 1: Amount of ochratoxin A, citrime, Zearalenone, Fumonisin, aflotoxin in pet food and tissues of cat

<table>
<thead>
<tr>
<th>samples</th>
<th>OTA (µg/kg)</th>
<th>CIT (µg/kg)</th>
<th>ZEA (µg/kg)</th>
<th>FB1 (µg/kg)</th>
<th>AFB1 (µg/kg)</th>
<th>Other AFBs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Petfood 1*</td>
<td>0.044</td>
<td>0.094</td>
<td>ND</td>
<td>81</td>
<td>0.006</td>
<td>ND</td>
</tr>
<tr>
<td>Blood mother 1</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>B2</td>
</tr>
<tr>
<td>Blood cat x</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>B2</td>
</tr>
<tr>
<td>Petfood 2</td>
<td>0.18</td>
<td>0.13</td>
<td>165</td>
<td>229</td>
<td>trace</td>
<td>Trace B2, G1, G2</td>
</tr>
<tr>
<td>Plasma mother 2</td>
<td>0.117</td>
<td>1.114</td>
<td>NA</td>
<td>19.75</td>
<td>0.464</td>
<td>B2</td>
</tr>
<tr>
<td>Liver kitten 2</td>
<td>ND</td>
<td>ND</td>
<td>13.66</td>
<td>ND</td>
<td>ND</td>
<td>Traces B1, B2</td>
</tr>
<tr>
<td>Kidney kitten 2</td>
<td>4.39</td>
<td>ND</td>
<td>NA</td>
<td>NA</td>
<td>ND</td>
<td>B2</td>
</tr>
<tr>
<td>Brain kitten 2</td>
<td>1.083</td>
<td>ND</td>
<td>91.33</td>
<td>32.24</td>
<td>0.77</td>
<td>B2</td>
</tr>
<tr>
<td>Intestine kitten 2</td>
<td>0.63</td>
<td>ND</td>
<td>7.83</td>
<td>0</td>
<td>0.047</td>
<td>B2</td>
</tr>
<tr>
<td>Petfood 3</td>
<td>0.34</td>
<td>0.1</td>
<td>18</td>
<td>250</td>
<td>0.03</td>
<td>B2; G2</td>
</tr>
<tr>
<td>Blood mother 3</td>
<td>0.059</td>
<td>2.36</td>
<td>29.518</td>
<td>3.88</td>
<td>0.105</td>
<td>B2</td>
</tr>
<tr>
<td>Placenta kitten 3</td>
<td>ND</td>
<td>ND</td>
<td>67.14</td>
<td>23.12</td>
<td>0.308</td>
<td>B2</td>
</tr>
<tr>
<td>Liver kitten 3</td>
<td>ND</td>
<td>ND</td>
<td>71.05</td>
<td>79.84</td>
<td>0.749</td>
<td>G2, B2</td>
</tr>
<tr>
<td>Kidney kitten 3</td>
<td>ND</td>
<td>ND</td>
<td>79.05</td>
<td>87.23</td>
<td>0.492</td>
<td>B2</td>
</tr>
</tbody>
</table>

*Petfood 1 does not induce any cat health problem; petfood 2 and petfood 3 induced stillbirth.
From late autumn till spring, wild boars have mainly been feeding on mouldy cobs, contaminated with high levels of *Fusarium* metabolites, as a result of mass corn harvest and abundance of the plant. Zearalenone, in particular, may be responsible for hyperestrogenism observed in young sows. The aim of the study was to determine the level of *Fusarium* mycotoxins contamination of corn cobs and subsequently to check the content of the toxins in the digestive tract of wild boars, hunted in field (large corn fields) and forest areas (at times fed with cereal waste). The wild boars from the field and forest areas (n-14 and n-12, respectively) - the young sows (60/70 kg) were hunted for between November and December in kujawsko-pomorskie province.

*Fusarium* mycotoxins (trichothecenes and zearalenone) were evaluated using HPLC-MS/MS method (API 4000, AB Scieix) in the samples of corn cobs, corn and oats grain, stomach content and faeces. The samples were cleaned on the Bond Elut® Mycotoxin columns (Agilent). During the samples preparation isotopic marked, isotope labelled internal standards of DON, T-2 and HT-2 toxins and ZEN were applied. The results of the mycotoxicological examination, expressed as mean concentration, have been presented in the tables 1-3.

### Table 1: Mycotoxins content in the feed for wild boars from forest and field areas [ppb]

<table>
<thead>
<tr>
<th>Material</th>
<th>DON</th>
<th>NIV</th>
<th>3ADON</th>
<th>MAS</th>
<th>DAS</th>
<th>ZEN</th>
<th>T-2</th>
<th>HT-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Field areas</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>maize cobs</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>X/2011</td>
<td>405.0</td>
<td>&lt;3.0</td>
<td>28.4</td>
<td>nd</td>
<td>nd</td>
<td>58.5</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>XI/2011</td>
<td>1711.0</td>
<td>47.9</td>
<td>60.0</td>
<td>&lt;1.5</td>
<td>&lt;1.0</td>
<td>217.0</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>XII/2011</td>
<td>4106.0</td>
<td>154.0</td>
<td>411.0</td>
<td>3.1</td>
<td>&lt;1.0</td>
<td>762.0</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>III/2012</td>
<td>5639.0</td>
<td>312.0</td>
<td>19.2</td>
<td>5.9</td>
<td>nd</td>
<td>912.0</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>IV/2012</td>
<td>28607.0</td>
<td>1091.0</td>
<td>842.0</td>
<td>&lt;1.5</td>
<td>nd</td>
<td>750.0</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Forest areas</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maize</td>
<td>28.4</td>
<td>8.5</td>
<td>7.5</td>
<td>2.1</td>
<td>1.9</td>
<td>0.9</td>
<td>1.0</td>
<td>nd</td>
</tr>
<tr>
<td>Oat</td>
<td>47.3</td>
<td>2053.0</td>
<td>13.4</td>
<td>118.0</td>
<td>20.0</td>
<td>28.8</td>
<td>5.8</td>
<td>19.7</td>
</tr>
</tbody>
</table>

### Table 2: Trichothecenes and zearalenone levels in the stomach content of wild boars [ppb]

<table>
<thead>
<tr>
<th>Stomach content [ppb]</th>
<th>DON</th>
<th>NIV</th>
<th>DOM</th>
<th>MAS</th>
<th>DAS</th>
<th>ZEN</th>
<th>T2</th>
<th>HT2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Field areas</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>mean</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nd</td>
<td>5.0</td>
<td>nd</td>
<td>&lt;1.5</td>
<td>nd</td>
<td>17.2</td>
<td>nd</td>
<td>&lt;1.2</td>
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<tr>
<td>range</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nd-19.7</td>
<td>nd-32.6</td>
<td>nd-&lt;3</td>
<td>nd-1.7</td>
<td>-</td>
<td>nd-67.5</td>
<td>nd-2.9</td>
<td>nd-4.1</td>
<td></td>
</tr>
<tr>
<td>Forest areas</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mean</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nd</td>
<td>22.2</td>
<td>nd</td>
<td>4.0</td>
<td>nd</td>
<td>4.2</td>
<td>nd</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td>range</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nd-147.5</td>
<td>nd-210</td>
<td>-</td>
<td>nd-43.9</td>
<td>-</td>
<td>&lt;0.5-12.5</td>
<td>nd-1.3</td>
<td>nd-2.8</td>
<td></td>
</tr>
</tbody>
</table>

### Table 3: Trichothecenes and zearalenone levels in the faeces of wild boars [ppb]

<table>
<thead>
<tr>
<th>Faeces [ppb]</th>
<th>DON</th>
<th>NIV</th>
<th>DOM</th>
<th>MAS</th>
<th>DAS</th>
<th>ZEN</th>
<th>T2</th>
<th>HT2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Field areas</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>mean</td>
<td>2.2</td>
<td>&lt;1.5</td>
<td>3.2</td>
<td>nd</td>
<td>nd</td>
<td>13.3</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>range</td>
<td>nd-6.7</td>
<td>nd-2.9</td>
<td>nd-7.3</td>
<td>-</td>
<td>-</td>
<td>nd-63.2</td>
<td>-</td>
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<tr>
<td>Forest areas</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mean</td>
<td>3.3</td>
<td>5.0</td>
<td>&lt;3</td>
<td>nd</td>
<td>nd</td>
<td>4.0</td>
<td>nd</td>
<td>nd</td>
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<tr>
<td>range</td>
<td>&lt;1-10.6</td>
<td>nd-48.7</td>
<td>nd-6.5</td>
<td>-</td>
<td>-</td>
<td>&lt;0.5-19.7</td>
<td>nd-&lt;1</td>
<td>-</td>
</tr>
</tbody>
</table>

The results of the analysis of faeces and stomach content samples, confirm that mouldy corn cobs can be responsible for mycotoxicosis in wild boars (including hyperestrogenism).

**Acknowledgements**

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IMPACT ON ANIMAL AND HUMAN HEALTH – P106

Ochratoxin A Residues in chicken tissues in experimental ochratoxicosis

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Objectives: To observe the effects of age, dietary levels and duration of exposure of OTA upon its residues in liver and muscles of broiler chicks. In experiments 1 and 2, broiler chicks of one-day and 21 days of age were kept on feeds containing 0, 1.6, 3.2 and 6.4 mg/kg OTA for 10 days. While, in experiment 3 broiler chicks of one-day of age were kept on feeds containing 0, 0.05, 0.1, 0.2, 0.4 and 0.8 mg/kg OTA for 35 days, respectively.

OTA Residues in Liver: In experiment 1 OTA was detected on day 3 in all treatment groups. The residue concentration increased till day 11 and was 25.75±1.02, 31.78±0.75 and 171.19±3.37, respectively in 1.6, 3.2 and 6.4 mg/kg groups. Upon withdrawal of dietary OTA the residual OTA levels decreased from day 12 and could not be detected on day 18 in 1.6 and 3.2 mg/kg OTA groups while it became undetected in 6.4 mg/kg OTA group on day 21 of the experiment.
In experiment 2 OTA was detected in all treatment groups on day 3 of the experiment. It became undetectable on days 15, 18 and 21, respectively in 1.6, 3.2 and 6.4 mg/kg groups.

OTA Residues in Muscles: In experiment 1, the OTA residues were detected in all treatment groups on day 3. OTA levels on day 11 were 1.897±0.01, 1.876±0.01 and 4.985±0.18 ng/g, respectively. OTA residues decreased from day 12 and were undetectable on day 15 in 1.6 and 3.2 mg/kg groups while in 6.4 mg/kg group it was undetected on day 18 of the experiment.
In experiment 2 OTA was detected in all treatment groups on day 3 of the experiment. It became undetectable on days 13 in 1.6 mg/kg group and day 15 in 3.2 and 6.4 mg/kg groups.

In experiment 3 OTA was detected on day 5, 14, 21 and 35 in 0.8, 0.40 & 0.20, 0.1 and 0.05 mg/kg group, respectively. The highest levels were 0.200±0.03, 0.210±0.11, 1.126±0.31, 1.014±0.61 and 1.375±0.02, respectively on day 35 of the experiment. Upon withdrawal at day 35, the OTA residues became undetectable on day 39 in 0.1-0.2 mg/kg groups and on day 42 in 0.4 and 0.8 mg/kg groups.

Conclusions: Tissue concentration of OTA was consistently higher in liver than muscles. Tissue residues appeared earlier in broiler chicks fed higher levels of OTA and then those fed lower levels. OTA tissue residues disappeared earlier in the older birds compared with the younger ones. OTA was detected in liver of birds from all treatment groups; therefore, it can be a potential candidate diagnostic tool for confirmation of ochratoxicosis in birds suspected on the basis of clinico-pathological observations.
Contamination of feed with mycotoxins seems to be a difficult problem to solve. Preventive measures do not always bring the solutions, therefore other methods are intruded. The most promising approach is mixing additives in the feed. Most products to reduce the effects of mycotoxin contamination are registered as technological additives or feedstuff and are fairly easy to register. This results in a sprawl of new products sold as such or mixed in tailor made compound mixtures for specific purposes/companies. The legislation for technological additives does not require full traceability, as it is the case for veterinary drugs intended to be mixed in the feed. Often, the content of the feed, including mycotoxin binders and/or modifiers, is not fully known by the different parties involved in the value chain of these products. The sprawl of new and mixed products, the flexible legislation, the non-transparent use and the multiple and entangled roles of the different companies contribute to the problems of clearly defining the most relevant products. In order for further research on this topic, it would be necessary to include the most relevant products and ingredients.

This study aims to identify frequently used materials to detoxify mycotoxin contaminated feed by extensive interviews with industry experts and important stakeholders. Eleven producers of feed additives and three compound feed producers were contacted. Furthermore, experts from interest groups such as BEMEFA and FRANA, governmental organizations such as Federal Public Service for Health, Food chain safety and Environment, veterinarians and farmers were prepared to collaborate in this study. To differentiate the different products, the Cation Exchange Capacity (CEC), type of exchangeable cations, and pH will be determined for the different products and their ingredients. The CEC stands for the maximum amount of bases the feed additive can exchange for other substances such as nutrients or mycotoxins and will be determined by quantitative ammonium distillation (Rhoades et al., 1982; Van Ranst et al., 1999). The type of cations permits further differentiation between samples and will be determined by Inductive Coupled Plasma (ICP) analysis of the ammonium extract (Anonymous, 2010). Results will be presented at the Workshop.

Acknowledgements
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Compendium for sampling, measurements and analysis of water (WAC), version WAC/III/B/010:10/2010
Fumonisins are toxic secondary metabolites produced by *Fusarium* spp. on maize. Unfortunately, fumonisins are highly stable molecules and their detoxification is poorly suitable for industrial processing. Considering the worldwide contamination of corn by *Fusarium* species in the field, and that fumonisins are produced before harvesting, one promising option for reducing the mycotoxic risk could be the degradation of fumonisins by using antagonist microorganisms isolated directly from plant microbiota before feeding livestock. Moreover, experimental evidence showed a fumonisins level decrease in some analysed samples during the moist corn grain silage fermentation. Therefore, the aim of this work is to select an endemic microbial consortium of moist corn grain silage that could be able to transform fumonisins in non-toxic products.

To address this objective, functional screening using classical methods and high-resolution metagenomics approaches will be performed. To achieve our goals, three main questions will be answered.

1- Are fumonisins actually degraded and detoxified or only adsorbed and not available for conventional analyses methods? Analytical methods combining various extractions procedures and fine HPLC-MS analyses of the mycotoxins and their degradation products are under development.

2- Are there any differences in microbial community composition in moist corn grain silages leading or not to significant fumonisin degradation? Comparison of the microbial consortia and metagenomes of contrasted moist corn grain silages samples at different time is a valuable source of data on this unexplored microbial ecosystem and of degrading inocula.

3- What are the detoxification mechanisms and the microbial activities involved? It will be expected to identify “detoxification genes” to be used as markers of detoxifying silages that could be used as a predictive and a screening tool to target degrading microbes.
Protein expression of two ergot alkaloid degrading enzymes in *Pichia pastoris* and *Escherichia coli*

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Ergot alkaloids are mycotoxins mainly produced by *Claviceps* species in cereals such as rye, sorghum, wheat and oat and by *Neotyphodium* species in grasses. Intoxication of animals is a major medical and economical issue.

Two enzymes, an alpha/beta-hydrolase (ErgA) and an amidase (ErgB), were isolated from an ergot alkaloid degrading strain, *Rhodococcus erythropolis* MTHt3, and found to be able to degrade ergopeptines to lysergic acid, with the lysergic acid amide ergine as intermediate. For the use of the enzymes as feed additives for degradation of ergot alkaloids in the gastrointestinal tract of animals a suitable expression host for enzyme production is required.

Two expression systems, *Pichia pastoris* CBS7435 and different DE3 strains of *Escherichia coli*, were used. Yields of protein expression of both enzymes were analysed by SDS-PAGE and enzymatic activity of expressed protein was measured by HPLC and fluorescence detection.

The ergopeptine-degrading ErgA could be expressed in *E. coli* ArcticExpress. Of the total protein produced, 50% was soluble. The enzyme was also expressed in *P. pastoris*. However, the yield of protein was lower compared to *E. coli* ArcticExpress. ErgA expressed in *E. coli* ArcticExpress showed better activity in the reaction degrading ergotamine to ergine than the same protein expressed in *P. pastoris*.

For the expression of the ergine-degrading ErgB different expression strains of *E. coli* were compared. In *E. coli* Rosetta 2, HMS174, BL21 and Tuner the protein was expressed but with low solubility varying between 0 and 10%. It seemed that the expressed protein had negative influence on cell growth of *E. coli* ArcticExpress and as a consequence, no enzyme could be gained. In *E. coli* BL21pLysS, a strain for the expression of toxic compounds, no protein was expressed. In the reaction degrading ergine to lysergic acid no enzymatic activity was measured with expressed protein.

For further experiments a different cloning strategy in *P. pastoris* might improve expression yield of ErgA. Various expression systems might be useful for the expression of ErgB.
REDUCTION AND PREVENTION – P110

Development of microbial formulation against mycotoxigenic fungi

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The mould contamination, particularly with mycotoxin-producing fungi is a global problem. Approximately, 25% of the world crops are affected by mycotoxins annually. Mycotoxins create a serious problem for animal and human health and have a significant economic effect worldwide (Dimitrious et al. 2012). The problem of fungal contamination of foods and feeds in India is very common. The most economically damaging mycotoxin are aflatoxins (Ahmad et al. 2013) which are highly toxic, mutagenic, immunosuppressive and carcinogenic to animals (Abdin et al. 2010). Aflatoxins contamination of food and feed presents a serious food safety issue on a global scale, causing tremendous yield and economic losses. Current agricultural practices emphasize on environmental sustainability by limiting the use of chemical fertilizer and pesticides. Soil bio-amendments offer promising alternatives to minimize the deleterious effects of chemical fertilizers. Encapsulation of living cells in polymeric gel is a well established technology in a broad and increasing range of different applications. The gel-like matrix allows the cells to remain viable and with its catalytic ability for longer duration (Ahmad et al. 2012 ).The development of biocontrol formulation against mycotoxigenic fungi forms one of the strongest alternative and since the antagonistic activity of large number of bacteria has been found out to be effective against the mycotoxigenic fungi.

The main objective of this study was to select such microbes that show antagonism towards mycotoxigenic fungi and hence to immobilize them as biocontrol agent within the alginate beads and hence to study their long term storage properties. A pre-isolated bacterium (Bacillus sp. SJ604) from our laboratory known for chitinase production was used to study the interaction with mycotoxigenic fungi (Aspergillus flavus MTCC 277). Therefore, a method of batch fermentation of immobilized bacterial cells was developed to enhance its secretion and increase the bacterial shelf-life. Optimization conditions suggested the alginate concentration of 4% at a pH range of 6.0-7.0 and temperature of 37 °C are the best for achieving maximum antagonistic activity by immobilized cells. Amendment of alginate with 5% skimmed-milk powder added greater stability, and also maintained the viability of bacterial cells inside the entrapped alginate beads. Thus, the immobilization resulted as nutrient source for maintaining the critical biomass with enhanced stability during long term storage at ambient temperature for providing a longer shelf-life.

References:
Role of essential oils in the reduction of Aflatoxin B₁ in maize grain

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Aflatoxins (AFT) are secondary metabolism which is produced by variety of food borne fungi like Aspergillus flavus, A. parasiticus and A. nomius. They infect wide variety of grains like maize, peanut, wheat, etc. Essential oils, a secondary metabolism of plants, have been used as potential antimicrobial agent against various fungi. In the present study, 8 kinds of essential oils namely Anise, Camphor, Cinnamon, Citral, Eucalyptus, Eugenol, Litsea cubeba, and Poleo were used to analyze their fumigation effect and their impact in the reduction of aflatoxin B₁ (AFB₁). On the initial screening of the oils, Cinnamon oil proved to be effective in degrading AFB₁ in the contaminated maize from 49.79 µg/kg to 27.90 µg/kg. When the fumigation time was extended to 28 days there was 71.75% of reduction in AFB₁. On further increasing of the days above 28 days didn’t show any change in the reduction of AFB₁. The present study suggests that Cinnamon oil could be a potential fumigating agent in the control of Aspergillus sp and also in the reduction of Aflatoxins.
**REDUCTION AND PREVENTION – P112**

**The influence of NaHCO3 on thermal reduction of aflatoxins, fumonisins, deoxynivalenol, nivalenol and zearalenone in maize flour during baking**

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The stability of naturally occurring aflatoxin B1 (AFB1), AFG1, fumonisin B1 (FB1), FB2, FB3, zearalenone (ZEA) nivalenol (NIV), deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON) and beauvericin (BEAU) was evaluated during a Malawian traditional maize cake process on a laboratory scale. Two different cake recipes were tried out: the first recipe involved the use of maize flour, NaHCO3 (soda) and water while the second recipe involved the use of maize flour and water only. Both cakes were baked at a constant temperature of 200 degrees Celsius for 1 hour. Liquid chromatography-tandem mass spectrometry was used to determine the mycotoxin levels in the flour and cake samples. Reduction of FB1, FB2, FB3, ZEA, DON, 3-ADON and 15-ADON ranged from 5 to 62 percent and 71 to 95 percent in non-soda and soda cakes respectively. AFB1, AFG1 and NIV reduction ranged from 19 to 52 percent in non-soda cakes and were not detected in soda cakes. Concentrations of all the mycotoxins in the soda cakes were significantly (probability value less than 5 percent) lower compared to non-soda cakes. This is a first report on the effect of NaHCO3 on these mycotoxins during the baking of maize cake.
REDUCTION AND PREVENTION – P113

In vitro antifungal activities of essential oil from Nigerian medicinal plants against toxigenic Aspergillus flavus

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The aim of this study was to determine the sensitivity profile of toxigenic mould strain isolated from poultry feed to some essential oils. Essential oils extracted from eight medicinal plants collected from South-West Nigeria were screened for their activity against toxigenic Aspergillus flavus by disc diffusion method. Minimum inhibitory concentrations of oils against Aspergillus flavus were done by agar dilution method and minimum fungicidal concentration data were obtained by the broth micro dilution method. Different concentrations of the essential oils were also tested for spore germination of the assayed mould strains. In addition, the essential oils were able to inhibit the mould spores germination when assayed at different concentrations. The results showed that the maximum antifungal activity, minimum inhibitory concentration and reduction in spore germination was demonstrated by oils of Cymbopogon citratus, Allium sativum and Ocimum basilicum as compared to control, which showed activity similar to control (miconazole nitrate). The essential oils of Zingiber officinalis, Citrus limon and Citrus aurantifolia exhibited moderate activity. The oils of Citrus sinensis demonstrated comparatively low activity against the isolate as compared to control while the oil of Pimenta racemosa did not show any activity against the isolate. These results shows that plant oils can be used to cure infections caused by toxigenic Aspergillus flavus and plant oils may have role as pharmaceuticals and preservatives.

![Graph showing the diameter of zone of inhibition of different essential oils](image)

Fig 1: Diameter of zone of inhibition of the essential oil of the extracts at 100% concentration

Deoxynivalenol (DON), a secondary metabolite produced by *Fusarium* species, is a lasting contaminant of feedstuff, which affects health and performance of farm animals. The bovine strain Eubacterium BBSH 797, which was isolated of rumen fluid, transforms DON into the less toxic metabolite de-epoxy-deoxynivalenol (DOM-1) (Fuchs et al., 2002). For that reason, Eubacterium BBSH 797 is used as feed additive to counteract the toxicity of DON. The present experiment compares the effect of DON and DOM-1 on cell viability, nitric oxide (NO) and cytokine production of murine macrophages (RAW 264.7) with and without lipopolysaccharide (LPS) stimulation. Stimulated (1000 ng/ml LPS) and non-stimulated cells were incubated with DON (7.8 – 250 ng/ml) and DOM-1 (500 – 8000 ng/ml) for 24 hours. Cell viability was monitored with WST-1((4- [3 - (4 -iodophenyl)-2- (4 -nitrophenyl)- 2H -5-tetrazolio]- 1,3 -benzene disulfonate). NO production in the cell culture supernatant was determined by Griess Reagent. The cytokines interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-alpha) were determined in the cell culture supernatant by a bead-based flow cytometric assay (FlowCytomix™). DON at 250 ng/ml reduced cell viability up to 40 %, DON at 125 ng/ml up to 12 %. Lower DON concentrations did not reduce cell viability. DOM-1 did not affect cell viability up to 8000 ng/ml. LPS-stimulated NO production was reduced up to 71 % at 250 ng/ml DON, and up to 16 % by 125 ng/ml DON. DOM-1 did not affect LPS-stimulated NO production even at the highest concentration tested (8000 ng/ml). DON and DOM-1 without LPS did not stimulate NO production. DON and DOM-1 did also not stimulate IL-6 production. While DON superinduced LPS-stimulated IL-6 production concentration-dependently, DOM-1 showed no concentration-dependent effect. In contrast, DON without LPS stimulated TNF-alpha production concentration-dependently and showed no concentration dependent effect on LPS-stimulated TNF-alpha production. DOM-1 showed no concentration-dependent effect on TNF-alpha production with and without LPS. To sum up, DON reduced cell viability, decreased LPS-stimulated NO production, superinduced LPS-stimulated IL-6 production and stimulated TNF-alpha production. Even the 32-fold concentration of DOM-1 (i.e. the highest concentration tested) showed none of these effects. The results confirm that DON is detoxified by its transformation to DOM-1 and support the use of Eubacterium BBSH 797 as feed additive.

References
Effect of deoxynivalenol and de-epoxy-deoxynivalenol on albumin production by HepG2 cells

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The trichothecene mycotoxin deoxynivalenol (DON) is a frequent contaminant of feed, causing impaired growth in animals. Some intestinal bacteria, such as the bovine strain Eubacterium BBSH 797, are capable of transforming DON to the less toxic metabolite de-epoxy-deoxynivalenol (DOM-1). Therefore, Eubacterium BBSH 797 qualifies as a valuable feed additive for the purpose of DON detoxification. As the inhibition of protein synthesis is considered one of the primary toxicity mechanisms of DON, reduced albumin production indicates potential cytotoxic effects of a substance. The effect of DON and DOM-1 on the albumin production of the human hepatocellular liver carcinoma cell line HepG2 was compared. Cells at approximately 80% confluence were incubated with DON (62.5–64,000 ng/ml) or DOM-1 (62.5–64,000 ng/ml) for 24 h. Albumin production of DON and DOM-1 treated HepG2 cells was determined via Enzyme-linked Immunosorbent Assay (ELISA). In parallel, cytotoxicity of DON and DOM-1 was monitored via metabolic activity using WST-1((4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate). HepG2 mediated albumin production decreased considerably with increasing DON concentrations, which is a sign for the disturbance of cellular functions by DON. Between 500 and 64,000 ng/ml DON, albumin production by HepG2 cells was reduced by over 50%. In contrast, DOM-1 had no such effect on albumin production between 62.5 and 64,000 ng/ml. Albumin production of DOM-1 treated HepG2 cells was not reduced to or below 50% at any test concentration. This effect is in accordance with viability data. In contrast to DON, DOM-1 had no negative effect on the viability of HepG2 cells at any test concentration. In summary, while DON considerably compromised albumin production at and exceeding 500 ng/ml, the effect of DOM-1 on HepG2 albumin production was far less drastic. The data therefore confirms the detoxification mechanism of Eubacterium BBSH 797 and support its use as a DON detoxifying feed additive.
REDUCTION AND PREVENTION – P116

Effects of increasing concentrations of sodium sulfite on deoxynivalenol concentrations of maize meal and maize kernels preserved with propionic acid at various moisture contents

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The mycotoxin deoxynivalenol (DON) formed by field fungi of the genus Fusarium might affect animal health by the consumption of contaminated feeds. Under European climatic conditions DON contamination occurs frequently on cereals, including maize. Detoxification measures are required if critical DON concentrations are exceeded in order to avoid adverse effects on farm animals. It was reviewed recently that a wet treatment of DON contaminated cereal grains with sodium metabisulfite results in a reduction of the DON concentration and a concomitant increase in DON sulfonate which is regarded as a detoxified derivative of DON. However, it was not investigated so far if the alternative substance sodium sulfite also leads to such DON decreasing effects.

Hence, the present study aimed to investigate the kinetics of DON concentration in maize kernels and maize meal, contaminated with 46.389 mg DON per kg dry matter, during wet preservation with sodium sulfite (Na₂SO₃) and propionic acid. To find the optimum Na₂SO₃ - dose for maximum DON reduction and to examine the interaction between dose and moisture content in dependence on the preservation duration, increasing Na₂SO₃ dosages (0, 1.25, 2.5, 5 and 10 g per kg) were tested at total moisture contents of 14% and 30% for up to 79 days. Propionic acid was included in all treatment variants at a constant dose of 15 g per kg to avoid microbial spoilage.

In general, DON concentration decreased with increasing amounts of supplemented Na₂SO₃ and with increasing duration of the preservation period in a bi-exponential fashion when Na₂SO₃ addition was ≥ 2.5 g/kg. At lower additions of Na₂SO₃ only minor and inconsistent DON reduction rates were observed. All variants preserved at 30% moisture led to high DON reduction rates after 79 days. The measured DON concentration in variants supplemented with 10 g Na₂SO₃ per kg amounted at 30% moisture to 4 and 3% of the initial concentration for kernels and meal, respectively while the corresponding recovery for the variants preserved at 14% amounted to 15 and 42%, respectively. Thus, after 79 days, a slight reduction rate of DON in maize meal at 14% was visible. This observation could be explained by the greater surface texture of the feed and the predisposing factor to the higher moisture content. In conclusion, the overall results and statistical analysis clarified that the highest Na₂SO₃ addition of 10 g per kg of maize at a moisture content of 30% for 79 days might be necessary to obtain a maximum DON reduction. Here, the feed structure and moisture content had a significant influence on the decontaminating effect of sodium sulfite.
**Abstracts of posters**

35th Mycotoxin Workshop  
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**TOXICOLOGY – P117**

Different mechanisms of damaging DNA by Altertoxin II

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In the early 1960s an association between the consumption of food contaminated with *Alternaria* and an increased incidence of esophageal cancer was observed in the region Linxian in China (Dong et al. 1987). A broad spectrum of secondary metabolites is produced by *Alternaria* species. Yet, 30 of 120 known substances are suspected of being toxic (Bottalico and Logrieco 1992), thus representing a potential risk for the health of humans. However, as summarized recently in an opinion of the European Food Safety Authority, the data concerning the toxicity of *Alternaria* spp. and their toxins are limited (CONTAM 2011). We succeeded recently to identify altertoxin II (ATX-II) as a genotoxic impact compound in complex extracts obtained from rice infested with *Alternaria alternata* (Schwarz et al. 2012b). In contrast, alternariol (AOH), one of the major *Alternaria* toxins with respect to quantity, was found to contribute to genotoxic properties of complex extracts only to a marginal extent (Schwarz et al. 2012a). The DNA-damaging properties of AOH could be at least partially attributed to the interference with DNA-topoisomerase II (Fehr et al. 2009; Fehr et al. 2010). In the present study we addressed the question whether topoisomerase targeting is a common feature of *Alternaria* toxin and whether also ATX-II acts as a topoisomerase II poison. Impact on topoisomerase II activity was investigated using the decatenation assay, a cell-free test system. Subsequently, effects on the stability of covalent topoisomerase II-DNA intermediates were analysed in cell culture with the *in vivo* complex of enzyme (ICE) assay. In parallel, impact on the level of intracellular redox systems was measured with the dichlorofluorescein assay (DCF).

In the comet assay ATX-II revealed a significantly higher DNA strand breaking potency in HT29 cells than AOH with substantial induction of fpg-sensitive sites, not to be observed after incubation with AOH. The results of the decatenation assay showed a clear inhibition of topoisomerase II at 33 µM ATX-II. However, under cell culture condition substantial cytotoxicity was already apparent in that concentration range. In the sulforhodamine B assay (SRB) after 24 h of incubation an IC50 value of 10 µM ATX-II was observed. Hence, no effects were detected in the ICE assay at 10 µM after 1h of incubation. Furthermore, ATX-II showed no increased formation of reactive oxygen species (ROS) measured with the DCF assay.

In conclusion, ATX-II indeed showed potency for targeting of topoisomerase II, however in contrast to AOH no topoisomerase poisoning was observed so far and DNA damaging was already present in a lower concentration range than topoisomerase targeting. Thus the present data argue to the fact that topoisomerase targeting and oxidative stress do not play a decisive role for the genotoxic properties of ATX-II. It might be speculated that the enhanced levels of fpg-sensitive sites result from the formation of DNA-adducts. Respective studies are currently in progress.

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Cytotoxicity of grain, their by-products and feed contaminated with Penicillium spp.

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Fungi growing under different environmental conditions strengthen their properties that enable them to establish in the particular environment, to develop and prevent growth of competitors. For such purposes many fungi produce and excrete toxic secondary metabolites. Penicillium expansum, P. palitans, P. viridicatum and other Penicillium species, widespread in various countries and particularly in Europe, could be an example. In Northern Europe ochratoxins are mainly produced by the Penicillium spp. Ochratoxins especially ochratoxin A are ubiquitous fungal metabolites known for their nephrotoxicity, carcinogenicity, genotoxicity, and immunotoxicity. Ochratoxins have been shown to be immunosuppressive in vivo and in vitro.

The aim of this study was to investigate the toxic effects of grain, their by-products and feed contaminated with Penicillium spp. secondary metabolites - ochratoxins.

L-41, Hep-2, K-562, Nb2-11, MH-2A cell lines were maintained under standard conditions in RPMI Medium 1640 w/glutamine or in DMEM supplemented with 10% FBS and antibiotics. Naturally contaminated grain and feed, grain, bran contaminated with Penicillium expansum, P. palitans, P. viridicatum were purified after extraction acetonitrile/water (84/16). Samples extracts were purified using the MultiSep # 212 cleanup column for ochratoxins. The clear extracts were evaporated in a Romer-Evap™ system and the residues were dissolved in cell medium and incubated in triplicates as two-fold dilution series with cell lines on a 96-well microtiter plate for 24 h. Cells were seeded at a density of 1x10⁻³ – 1x10⁻⁴ cells/ml. The viability of cell population and cell proliferation were examined by using the crystal violet and MTT assay. Cells viability was assayed spectrophotometrically using a Multiskan MS photometer (Finland). The cytotoxicity of ochratoxins was determined by measuring different endpoints such as inhibition of protein and DNA synthesis, plasma membrane integrity and reduced metabolic activity. The IC₅₀ value (the concentration of each sample reducing the total response to a 50% value of untreated cells) for cytotoxic compound was calculated.

About 50% of the SPEV and K-265 cells were obviously dead after exposure to ochratoxins concentrations 20-23 ng/ml in samples extracts. Barley contaminated OT concentration 67.4 mg/kg and 117.9 ng/ml cell culture medium, caused 87.5% of Hep-2 cell apoptosis.
Structure relationship of enniatin analogues, H, I and MK1688 in cytotoxic activity against human prostate cancer cells

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Fusarium mycotoxins, enniatins act as ionophores by forming dimeric “sandwich” structures that transport monovalent ions across membranes, particularly mitochondrial membrane and uncouple oxidative phosphorylation (Ivanov et al., 1973; Benz et al., 1978). Recently, it was shown that enniatins inhibit one of the major multidrug efflux pumps such as Pdr5p in Saccharomyces cerevisiae cells at non-toxic concentrations (Hiraga et al., 2005). The inhibition mechanism is clearly different from their function as ionophores (Hiraga et al., 2005). This property of the enniatins may be important for the industrial use in combination with chemotherapeutic drugs.

Recently, new three enniatin analogues, such as enniatins H, I and MK1688 were characterized from Fusarium oxysporum KFCC11363P isolated from fungi-contaminated potatoes in Korea. These new enniatin analogues have shown various biological properties including cytotoxicity, inducing of apoptosis and inhibition of human immunodeficiency virus type-1 integrase. Interestingly, these enniatin analogues exhibited different biological functions even though they show similar cyclic depsipeptide structure. Therefore, the aim of this study was to purify enniatins H, I and MK1688 from F. oxysporum KFCC 11363P submerged cultures in order to investigate their cytotoxicity against human prostate cancer cells. For this purpose the in vitro cytotoxic effect of enniatins H, I and MK1688 was evaluated on human prostate cancer lines (LNCaP, DU145 and PC3) using (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. This study may explain the relationship between structure and cytotoxic activity of enniatin analogues.

Enniatins H, I, and MK1688 contain an alternating sequence of three N-methyl-L-valine moieties [enniatin H, two D-hydroxyisovaleric acid (HIV) residues, and one hydroxymethylpentanoic acid (HMP) residue; I, one D-HIV, and two HMP residues; MK1688, three HMP residues] in their molecular structures. Enniatin MK1688 inhibited the growth of cancer cells lines most strongly and the cytotoxicity of enniatin MK1688 was approximately 2-fold higher than that of enniatin H. The cytotoxic effects of enniatin analogues on tested cancer cell lines significantly could be ranked in the following order: MK1688 > I > H. This study has shown that enniatins H, I, and MK1688 exhibit a strong in vitro cytotoxicity against human prostate cancer cells and the varying cytotoxic effects may be the result of conformational changes in their molecular structure.

References


Toxicity of the mycotoxin citrinin and its metabolite dihydrocitrinone

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Citrinin (CIT), a mycotoxin contaminant detected in various food commodities and in animal feed, got recently into the focus of the European Food Safety Agency [1]. It is considered as risk factor for human health since CIT is known to exert nephrotoxicity in several animal species, and it has genotoxic properties. But, a risk assessment for CIT is hampered by limitations in the toxicological database, and major deficits in exposure assessment [1]. Another open question is the metabolism of CIT in humans. Recently, we could demonstrate the frequent presence of this mycotoxin in human blood, and along with CIT the occurrence of dihydrocitrinone as a major metabolite in human urine [2]. These findings indicate that measurable amounts of CIT are ingested with the normal diet, and metabolized to dihydrocitrinone. Previously, this metabolite was detected in rats [3], but not in humans or other species. We have now studied the cytotoxic and genotoxic potential of this metabolite in comparison to the parent compound CIT.

Cytotoxicity was determined in V79 hamster fibroblast cells with the neutral red uptake assay: compared to CIT (IC\textsubscript{50} of 70 µM), dihydrocitrinone (IC\textsubscript{50} of 320 µM) caused distinctly lower cytotoxicity after treatment of cells for 24h. Genotoxicity was studied in V79 cells by means of the micronucleus assay: CIT induced a concentration-dependent increase in micronuclei frequencies at concentrations \( \geq 30 \) µM, whereas dihydrocitrinone showed no genotoxic effect up to 500 µM. These data show that the metabolite exhibits a much lower toxic potential than the parent compound. Thus, the conversion of CIT to dihydrocitrinone in humans can be regarded as a detoxification step. Our new results raise further questions regarding the enzymatic processes involved in mammalian citrinin metabolism.

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**References**

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

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To understand the cellular response during a confrontation with deoxynivalenol, a translation inhibitor, we screened a BY4741 yeast deletion library harbouring approximately 4200 strains carrying mutations of all nonessential genes, to identify genes whose deletion causes enhanced sensitivity to DON. Genetic display, in contrast to gene expression analysis, offers a direct assay close to the biological effect of a drug. The strain collection was modified by deletion of the PDR5 gene, encoding the most effective drug efflux pump in S. cerevisiae. This was done by systematic genetic techniques, generating a library more sensitive to drug treatment. We found 114 mutant strains displaying enhanced sensitivity to DON. We identified several clusters of cellular mechanisms potentially opposing DON toxicity and thus point to additional detrimental functions. These are DNA- damage, endocytosis, and cell cycle dependent transcription. In addition, we identified 56 mutants displaying higher resistance to DON. Gene expression analysis of DON treated cells complements the picture of cellular compensation responses and will be comparatively discussed. Comparison of DON with cycloheximide treatment shows that DON does not induce a generic response to inhibition of translation but induces a highly specific response pattern.
TOXICOLOGY – P123

S-methyl-DON: synthesis, structure, determination and toxicity tests of a novel mycotoxin conjugate

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S-Methyl-DON (SMD) is a novel plant metabolite of DON first described in Fusarium infected barley and wheat (Kushalappa et al., 2010). Our hypothesis is that SMD is derived from a DON-cysteine adduct, which is the product of processing of a DON-glutathione adduct. The DON-cysteine adduct was recently demonstrated to occur in DON treated wheat (Kluger et al., 2012). The aim of this study was to synthesize SMD for structure determination, as reference substance for quantification, and for toxicity tests.

The reaction conditions for the methylthio addition (Michael reaction) were optimized using model compounds for the acceptor functionality in DON. Finally, iodomethane (MeI), thiourea and sodium carbonate in wet polyethylene glycol 200 (PEG 200) were used for the synthesis of the desired product. The structure of the purified main product was confirmed using 2D-NMR-methods and NOE experiments. The resulting SMD not only showed the addition of a methylthio group to DON, but also an intramolecular hemiacetal was formed (scheme 1).

Scheme 1: Synthesis of SMD

The synthesized SMD was used to obtain preliminary toxicity data using an engineered toxin sensitive Saccharomyces cerevisiae stain. The SMD concentration required for 50% growth inhibition was about 9-fold higher than for DON. In addition, testing the inhibition of protein synthesis of wheat ribosomes (using a wheat germ in vitro translation system) showed that SMD is (17-fold) less toxic than DON. This indicates that the addition of the bulky cysteine and glutathione substituents should also lead to DON detoxification in planta.

References
Alternariol and Altertoxin II act as possible inducers of the AhR pathway

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Several mycotoxins formed by Alternaria alternata are widespread contaminants and relevant to the spoilage of food and feed (Li and Yoshizawa 2000). They are known to possess genotoxic properties thus representing a potential risk for human health. Especially alternariol (AOH) and altertoxin-II (ATX-II) have been recently reported to induce DNA strand breaks in mammalian cells already after 1h of incubation (Fehr et al. 2009; Schwarz et al. 2012). However, the different modes of action for both toxins are not completely elucidated yet.

In the present study we addressed the question whether the toxins activate the AhR pathway thus modulating the transcription of phase I enzymes. Therefore the impact on the transcription and protein level of cytochrome P450 enzymes (CYP1A1 and CYP1A2) in human esophageal cells (KYSE510) was investigated by quantitative real-time-PCR (q-PCR) after 24 h of incubation.

To exclude artifacts, cytotoxicity was determined by WST and SRB assay. Furthermore the increased amount of transcripts of these enzymes were verified at the protein level with the 7'-ethoxyresorufin-O-deethylase (EROD) assay to determine CYP activity.

The onset of cytotoxicity was observed after 24 h of incubation with 50 µM AOH, respectively. For ATX-II, a decrease of 30 % in cell viability was already detected at a concentration of 0.5 µM, which resulted in a different concentration range for AOH and ATX-II in the assays. Q-PCR revealed the highest induction of the transcription level of CYP1A1 at 10 µM AOH (10-fold), followed by 0.1 µM ATX-II (4-fold). To confirm these results, CYP activity was measured in the EROD assay. ATX-II enhanced CYP activity concentration-dependently up to 130 %, whereas AOH induced an increase above 150 %.

To conclude, incubation of KYSE510 esophagus cells with AOH or ATX-II leads to an increase in the transcription of CYP enzymes followed by enhanced CYP activity detected in the EROD-assay. Therefore it is conceivable that Alternaria toxins act as possible inducers of the AhR-Pathway.

Considering that AOH represents a potential substrate for CYP1A1, functionalization by enhanced CYP-activity might result in the formation of quinone structures thus potentially generating oxidative stress. Owing to the aromatic ring structure of ATX-II, it might also represent a potential substrate for CYP enzymes.

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Deoxynivalenol (DON) is a Fusarium mycotoxin and one of the most prevalent trichothecenes in nature. It has been associated with cellular death via inhibition of eukaryotic protein synthesis and alterations in cell signalling, differentiation and proliferation. Toxic effects of DON include emesis, haemorrhage, circulatory shock, resulting in implications to animal feeding behaviour and immune system function. The European Commission (EC) has set legislation limits for DON in cereal grains and cereal-based products intended for human consumption (EC No. 1126/2007). However, absence or low DON concentrations do not necessarily guarantee food safety, because it may still be present in its masked forms, making it undetectable by most analytical techniques. Deoxynivalenol-3-glucoside (D3G) is a phase II plant detoxification product of DON, found in naturally contaminated wheat, barley, oat and maize. Health implications related to the abundance of masked mycotoxins may not only be due to hydrolysis of conjugates to their precursor mycotoxins, but also due to their potential inherent toxicity. This study aims to provide some first toxicological data on the in vitro toxicity of D3G and in comparison to that of DON, as well as to examine its effects on the functional characteristics of human intestinal mucosa.

A widely used in vitro model of the human intestinal mucosa was based on the Caco-2 cell line clone C2BBe1 (human adenocarcinoma cells). The cells differentiate in 21 days to form an epithelium layer consisting of polarized enterocyte-like cells, with an apical brush border, morphologically comparable to that of the human colon. The cells were grown on Nunc\textsuperscript{TM} polycarbonate membrane-inserts (3 \textmu m pore size, 10 mm diameter) for 21 days. The integrity of the cell layer was tested using trans-epithelial-electrical resistance measurements and mannitol permeability. Hank’s balanced salt solution supplemented with 25 mM glucose, pH 7.4, was used as transport medium. All experiments were performed at 37°C. The inserts were washed and the test compounds DON and D3G (3-24 \textmu M and 6-48 \textmu M, respectively) together with mannitol were added on the apical side. Samples (100 \mu L) were collected at 30 min - 1 h intervals for 24 h from the basolateral side and will be analyzed with a novel, in-house validated, UPLC-Q-Tof-MS method utilizing amide chromatography. Cytotoxicity was assayed by means of metabolic activity (alamarBlue), DON and D3G concentrations ranged between 3-48 \mu M. Initial in vitro toxicity results for DON are in line with the literature with the CC\textsubscript{50} value for Caco-2 cells being 26 \mu M.

References
Inhibition of catechol-\(O\)-methyltransferase by genotoxic catechol metabolites of zearalenone

Erika Pfeiffer, Stefanie C. Fleck, Daniel Wefers, Andreas A. Hildebrand, Manfred Metzler

Zearalenone (ZEN) is an estrogenic mycotoxin produced by *Fusarium* species. The adverse effects of ZEN are often compared to those of 17\(\beta\)-estradiol (E2) and estrone (E1). These endogenous estrogens are associated with an increased risk for cancer, which may be mediated by two mechanisms, i.e. (i) hormonal activity and (ii) genotoxic effects by cytochrome P450-catalyzed metabolic activation to catechols [1]. Like E2 and E1, ZEN exhibits marked estrogenicity, undergoes aromatic hydroxylation to catechol metabolites [2] and also causes oxidative DNA damage under cell free conditions [3]. The subsequent methylation of catechols by catechol-O-methyltransferase (COMT) is generally considered to be a detoxifying pathway. The aim of this study was to investigate the \(O\)-methylation of the catechol metabolites of ZEN by hepatic COMT from human, mouse, rat, steer and piglet in comparison with the catechol metabolites of E2. The methylation products were analyzed by LC-DAD-MS/MS. By coincubating 13- or 15-HO-ZEN with 2-HO-E2, it was of interest to clarify whether they can influence the \(O\)-methylation of each other.

The individual catechols of ZEN, E1 and E2 are able to induce oxidative DNA damage in calf thymus DNA. The ranking of the DNA damaging activity was 15-HO-ZEN > 2/4-HO-E1/E2 > 13-HO-ZEN [3]. Concerning the detoxification pathway via methylation by human hepatic COMT, the ranking was 2-HO-E1/E2 >> 4-HO-E1/E2 > 13/15-HO-ZEN. The two catechol metabolites of ZEN are not only very poor substrates of human COMT but are also able to strongly inhibit the \(O\)-methylation of 2-HO-E2, the major catechol metabolite of E2. 15-HO-ZEN acts as a non-competitive inhibitor and is about ten times more potent than 13-HO-ZEN, which is an uncompetitive inhibitor of COMT. The catechol metabolites of ZEN were also shown to inhibit the \(O\)-methylation of 2-HO-E2 by hepatic COMT from mouse, rat and piglet, although to a lesser extent than observed with human COMT.

The mycoestrogen ZEN undergoes metabolic activation to reactive catechols with a DNA-damaging potential, in analogy to the steroidal estrogens E1 and E2. Compared with the steroidal catechol estrogens, all catechol metabolites of ZEN are poorer substrates for human COMT, and 15-HO-ZEN exhibits an even higher reactivity towards DNA. The powerful inhibitory effect of catechol metabolites of ZEN on COMT may have implications for the tumorigenic activity of E2, because catechol metabolites of E2 elicit genotoxic effects and their impaired \(O\)-methylation may increase the tumorigenicity of steroidal estrogens.

References


Aflatoxins are mycotoxins that contaminate feed and foods, considered by the Food and Drug Administration (FDA) as being inevitable contaminants (Kensler et al., 2004). Aflatoxin B1 is a very potent carcinogen in many species, including humans, birds, swine, fish, and rodents (Bennett & Klich, 2003; Kensler et al., 2004). AFB1 induces chromosomal aberrations, micronuclei, sister chromatid exchange, chromosomal strand breaks, and forms adducts in fish, birds and mammalian cells (IARC, 1993). On the basis of numerous evidence concerning its toxicity, AFB1 was classified in the group 1 of toxicity as a human carcinogen (IARC, 1993).

In this in vitro study, we compared the effects of different concentrations (0-100 microM) of aflatoxin B1 (AFB1) on two types of porcine cells: freshly isolated peripheral blood mononuclear cells (PBMC) and an epithelial intestinal cells line (IPEC-1), in term of cytotoxicity, inflammatory (IL-1 beta, TNF and IL-8) and anti-inflammatory cytokine (IL-10) synthesis and nitric oxide synthesis (NO).

After 48h of culture, different cytotoxic effects of AFB1 were observed on the two cell types, with PBMC cells being more sensitive to the toxic effects of the toxin, with a dose dependent decrease of cell viability, as showed by the MTT test. IPEC cells seems to be more resistant to the toxic effects of the AFB1.

Different effects were observed also concerning cytokine synthesis. Thus, in PBMC AFB1 induces an increase of the IL-8 synthesis by PMA-ionomycine stimulated cells (2.5 times for 100microM concentration). A slight increase was observed for IL-1beta and TNF alpha synthesis while no effect was observed for IL-10 synthesis or the NO synthesis.

In the IPEC cells, AFB1 induced a dose dependent decrease of the IL-8 synthesis, with the lowest value for the highest dose (58.47% decrease from control for the 100 microM concentration). AFB1 induces slight decrease of the synthesis of the two other inflammatory cytokines investigated: TNF-alpha and IL-1 beta. This decrease of the inflammatory cytokines was also associated with a dose dependent increase of the synthesis of IL-10 anti-inflammatory cytokine (28.44% higher than control for the 100 microM concentration) and a decrease of NO synthesis with 21.94% from the control.

These results demonstrate that AFB1 has a different effect on cell proliferation and cytokine synthesis in the two types of porcine cells analyzed, with important consequences for animal health.

References

Gene expression analyses of the impact of zearalenone on intestinal porcine epithelial cells (IPEC-1) in vitro

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Mycotoxins are fungal metabolites able to compromise the structural and functional characteristics of several tissues and organs in animals and humans, including the intestinal epithelium and the local immune response herein. The gut represents the main route of intoxication with mycotoxins. During an oral intoxication the gut epithelium is the first organ exposed and affected by different concentrations of mycotoxins. Zearalenone (ZEA) is a nonsteroidal estrogenic mycotoxin synthesized by a variety of Fusarium fungi, including F. graminearum (Gibberella zeae), F. culmorum, F. cerealis, F. equiseti, F. crookwellense and F. semitectum, which are common soil fungi, in temperate and warm countries, being regular contaminants of cereal crops worldwide. Most studies revealed the specific toxic effect of this toxin on the reproductive apparatus in laboratory and farm animals. In addition, ZEA has been shown to be immunotoxic in low concentrations. The pig, a great cereal consumer, is considered by EFSA the most sensitive species to ZEA action. Although the studies on ZEA intensified lately, the toxin has been little studied for its effect on the intestinal barrier and in terms of the potential link between exposure to mycotoxin and the intestinal inflammatory diseases (Maresca and Fantini, 2010). The mechanisms of its toxicity which include alterations of the expression of the genes associated to important metabolic processes are yet to be ascertained. Using a DNA microarray the aim of the present study was to evaluate the genome wide response of genes that are specifically expressed in response to low concentration of ZEA and to identify various pathways that control intestinal barrier function. After exposing intestinal porcine epithelial cells (IPEC-1) to 10µM of ZEA for 24h, genes expression profiles were analysed by microarray. Our results revealed that ZEA is a genotoxic mycotoxin able to modify the expression of a high number of genes implicated in various biological and molecular processes and the cellular components at intestinal level. 1645 modified genes were identified by the microarray analyses from which more than 70% were down-regulated and only 27% were up-regulated. The microarray results were validated by using real-time PCR. The effect of 10µM of ZEA on the relative expression of five different genes (IL-8, COX2, TLR1, TLR4, and STAT3) in IPEC-1 cells was analysed. A similarly up-regulation in the expression of selected genes was found like the one determined by microarray.
TOXICOLOGY – P129

Deoxynivalenol affects the composition of the basement membrane proteins and influences en route the migration of CD16-positive cells

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The basement membrane of the intestinal villi is perforated with numerous small pores. These pores are essential for the communication and interaction of enterocytes with cells in the lamina propria and vice versa. Deoxynivalenol (DON) is a trichothecene primarily produced by the plant pathogen Fusarium graminearum and F. culmorum and most prevalent in crops like wheat, oat or barley. DON contaminated products represent a serious problem in animal nutrition. It has been found that pigs are the most susceptible species and DON ingestion leads to reduced growth and thus to economical loss. The intestinal barrier is the main acting place of DON because it can act from both sides: basolateral and apical. On the basis of this background we analysed the effect of DON-contaminated feed intake in pigs with the focus on the composition, perforation and the immune system illustrated by migrated CD16-positive cells. To investigate the effect of DON on intestinal epithelial cells with the focus on the basement membrane we analysed DON-treated IPEC-J2 cells by microarray analysis.

Our results show that DON increases the pore number in the DON-treatment group of the jejunum probably via changing the composition of the basement membrane. This results in an increased migration of CD16-positive cells. On the other hand, DON affects the composition of the basement membrane shown by microarray analysis. Here, different genes are down-regulated like syndecan, BM-40 and fibulin which are important factors in the composition of the basement membrane. Other genes like MMP or TIMP which are important in the development and degradation are also regulated by DON. We suggest that DON acts in vivo from the basolateral side and has an effect the composition of the BM via influencing the protein production of fibroblasts and epithelial cells.
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Optimization of a GC-MS method for determination of type A and B-Trichothecenes in baby foods

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Trichothecenes (TRC) are a large group of mycotoxins, mainly produced by molds belonging to Fusarium genera. They are divided into four groups (types A, B, C and D) according to their characteristic functional groups. The most relevant in food and feed belongs to type A, such as T-2 toxin (T-2), HT-2 toxin (HT-2) and diacetoxyscirpenol (DAS), and type B, like deoxynivalenol (DON) and nivalenol (NIV) [1]. These toxins infect agricultural crops throughout the world, particularly cereals and cereal-based products and are recognized as a potential hazard for human and animal health, responsible for a wide range of toxic effects, such as immunosuppression, mutagenicity, neurotoxicity, gastrointestinal toxicity, anemia, etc. [2]. Despite their high toxicity, only DON has European Union legal limits.

The aim of this work was the optimization of an analytical procedure based on GC-MS for the simultaneous determination of 12 TRC, belonging to type A and B in baby foods commercialized in Portugal. In order to improve the derivatization step, various reagents were tested, with different reaction conditions: i) HFBA (heptafluorobutyric anhydride) at 80°C during 40 min; ii) TFAA (trifluoroacetic anhydride), 80°C 30 min; iii) PFPA (pentafluoropropionic anhydride) at 60°C for 60 min and iv) Tri-Sil TBT(BSA(bistrimethylsilylacetamide):TMSI(trimethylsilylimidazole):TMCS(trimethylchlorosilane),(3:3:2)) at 80°C from 30 min. The best results were obtained with the mixture of silylating agent (iv). Then, to lessen the time required for the derivatization step, a household microwave was tested at medium potency with different reaction times (3, 5 and 7 min). All reaction times tested are able to derivatize all the analytes, but the best signal was obtained with 3 min. The analysis is being performed by gas chromatography (GC) coupled to mass spectrometry (MS), after extraction with modified QuEChERS procedure.

In conclusion, it was successfully reached an improvement on derivatization step of trichothecenes, which allows the reduction of time spent in sample pre-treatment process before GC-MS analysis.

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