33rd Mycotoxin-Workshop

Conference Abstracts

Freising, Germany
30th May – 1st June, 2011

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Table of contents

Program

Monday, 30th May 2011

07.30 **Registration / Exhibition / Coffee**

09:00 **Opening of the 33rd Mycotoxin Workshop**
Prof. Dr. Hans-Ulrich Humpf, President of the Society for Mycotoxin Research
Rudolf Schwaiger, 2nd Bürgermeister of Freising
Prof. Dr. Dr. h.c. Johann Bauer, Technische Universität München, Hans Eisenmann-Zentrum

1. Session: Occurrence of mycotoxins I

9:30 **Down the drain: How humans emit deoxynivalenol into surface waters**
L01 F.E. Wettstein, T.D. Bucheli

9:45 **Influence of different water regimes on deoxynivalenol concentration in *Fusarium*-infected maize kernels**
L02 E. Oldenburg, S. Schittenhelm

10:00 **Impact of pre-harvest rainfall on *Fusarium* mycotoxin contamination of wheat**
L03 S. Edwards, L. Kharbikar, E. Dickin

10:15 **Coffee break / Exhibition**

2. Session: Occurrence of mycotoxins II

10:45 **Facts and figures: Masked *Fusarium* mycotoxins in cereals and cereal-derived products**
L04 M. De Boevre, J. Diana Di Mavungu, A. Versilovkis, P. Maene, K. Audenaert, A. Callebaut, G. Haesaert, M. Eeckhout, C. Van Peteghem, S. De Saeger

11:00 **Hidden fumonisins in maize hybrids and their correlation with chemical composition**
L05 C. Falavigna, C. Dall'Asta, G. Galaverna

11:15 **Fusarin C: Forgotten over 20 years?**
L06 K. Kleigrewe, A.-C. Söhnel, K. Hogrefe, P. Piecuch, H.-U. Humpf

11:30 **Mycotoxins in house dust - final results of a systematic study**
L07 Ch. Portner, V. Plegge, I. Toepfer, W. Butte

11:45 **Fate of mycotoxins during milling processes**
L08 S. Zühlke, S. Lehmann, B. Cramer, H.-U. Humpf, M. Spiteller

12:00 **Lunch / Exhibition**

13:00 **Poster Session I / Exhibition**

3. Session: Occurrence of mycotoxins III

14:30 **Occurrence of mycotoxins in beer brewed in Poland**
L09 A. Blajet-Kosicka, R. Kosicki, M. Twaruzek, J. Grajewski

14:45 **Ergot alkaloids: Determination and occurrence in EU foods and feeds**
L10 J. Diana Di Mavungu, D. Larionova, S. Malysheva, M. Sanders, J. Robbens, P. Dubruel, C. Van Peteghem, S. De Saeger

15:00 **Analysis of ergot alkaloids in cereal based food and feed from the 2006-2009**
L11 **harvests by HPLC/FLD and LC/MSMS**
S. Kemmlein, Ch. Gottschalk, H. Klaffke, C. Fauhl-Hassek

15:15 **Four years of wheat monitoring in Switzerland: Occurrence of different *Fusarium***
L12 **species and analyses of the factors influencing the mycotoxin production**
S. Vogelgsang, I. Bänziger, T.D. Bucheli, F. E. Wettstein, H.-R. Forrer

15:30 **Monitoring of Swiss commercial grain maize: Occurrence of *Fusarium* species,**
L13 **mycotoxins and the effect of cropping factors**
T. Musa, H.R. Forrer, S. Roffler, E. Jenny, S. Vogelgsang

15:45 **Coffee break / Exhibition**

16:15 **Meeting of the members of the Society for Mycotoxin Research**

18:30 **Walking tour to the "Bräustüberl" on Weihenstephaner Berg**
Meeting point in front of the congress center Kardinal-Döpfner-Haus

Tuesday, 31th May 2011

4. Session: Toxicology I

09:00 L14 **New aspects regarding deoxynivalenol in chicken**
A.W. Yunus, W.A. Awad, K.M. Ghareeb, J. Grajewski, J. Böhm

09:15 L15 **Effect of deoxynivalenol (DON) on health and performance of laying hens of different genetic background**
M. Ebrahem, S. Döll, S. Dänicke

09:30 L16 **Immunotoxicological effects in progeny of hens fed aflatoxin and ochratoxin contaminated rations**
M.Z. Khan, Z.U. Hassan, A. Khan, M.K. Saleemi, I. Javed

09:45 L17 **Use of different biomarkers to evaluate the toxicity of hydrolyzed fumonisin B1**
H. Schwartz, I. Schöner, O. Greitbauer, B. Grenier, R. Krska, G. Schatzmayr, F. Berthiller, I.P. Oswald, W.-D. Moll

10:00 L18 **New fumonisin metabolites: The *in vivo* formation of N-acyl-fumonisin B1 by the ceramide synthase**
H. Harrer, E.L. Laviad, H.-U. Humpf, A.H. Futermann

10:15 **Coffee break / Exhibition**

5. Session: Toxicology II

10:45 L19 **Trypacidin, a spore borne toxin from *Aspergillus fumigatus*, induces cytotoxicity in lung cells**
T. Gauthier, X. Wang, J.S. Dos Santos, A. Fysikopoulos, S. Tadrist, C. Canlet, M.P. Artigot, N. Loiseau, I.P. Oswald, O. Puel

11:00 L20 **Combined effects of deoxynivalenol and *Salmonella* Typhimurium on intestinal inflammation in the pig**
V. Vandenbroucke, F. Pasmans, A. Martel, E. Verbrugghe, J. Goossens, K. Van Deun, F. Boyen, P. De Backer, F. Haesebrouck, S. Croubels

11:15 L21 **Mycotoxin related cytotoxicity of grain products and production waste from grain mills in North-Rhine-Westphalia**
W. Föllmann, C. Behm, G.H. Degen

11:30 L22 **Functional genomic approach to examine the effects of *Fusarium* toxins on the immune response of pig: DNA microarray expression profiling in ileum/Peyer's patches**
B. Jakova-Strajn, A. Vengust, I. Ujcic-Vrhovnik, K. Fon Tacer

11:45 L23 **Effects of increasing concentrations of deoxynivalenol, zearalenone and ochratoxin A in diets for Atlantic salmon (*Salmo Salar*) on performance, health and toxin residues**
S. Döll, H. Valenta, G. Baardsen, P. Möller, W. Koppe, I. Stubhaug, S. Dänicke

12:00 **Lunch / Exhibition**

13:00 **Poster Session II / Exhibition**

6. Session: Analytical developments

14:15 L24 **Development and application of a stable isotope dilution assay for tenuazonic acid**
S. Asam, Y. Liu, M. Lichtenegger, M. Rychlik

14:30 L25 **Molecularly Imprinted Polymers (MIPs) towards twelve ergot alkaloids: Application in the clean-up of samples prior to LC-MS/MS analysis**
P. Lenain, J. Diana Di Mavungu, P. Dubruel, J. Robbens, C. Van Peteghem, S. De Saeger

14:45 L26 **Advanced synthesis of masked mycotoxins - methods and applications**
H. Mikula, Ch. Hametner, J. Fröhlich, F. Berthiller, R. Krska, G. Adam

15:00 L27 **Ochratoxin A metabolites in human blood and urine**
K. Munoz, B. Cramer, M. Blaszkewicz, H.-U. Humpf, G.H. Degen

15:15 L28 **UHPLC-MS/MS based method for the determination of mycotoxins regulated in the European Union using stable isotope labelled standards**
E. Varga, K. Mayer, T. Glauner, M. Sulyok, R. Schuhmacher, R. Krska, F. Berthiller

15:30 L29 **New insights into multi-mycotoxin analysis by LC-MS/MS**
M. Sulyok, B. Warth, V. Vishwanath, R. Schuhmacher, R. Krska

15:45 L30 **C8-2'-deoxyguanosine OTA-adducts detection and OTA metabolites in biological fluids as biomarker of human OTA exposure**
A. Pfohl-Leszkowicz, M. Tozlovanu, V. Faucet-Marquis, F. Pont, V. Stefanovic, R.A. Manderville

16:00 **Coffee break / Exhibition**

16:30 **Guided tour of the old town** *(Meeting point in front of the congress center Kardinal-Döpfner-Haus)*
Tour of Domberg/Cathedral hill *(Meeting point inner courtyard of the Kardinal-Döpfner-Haus)*

19:00 **Departure to the conference dinner at "Schuhbauers Tenne", Kirchdorf**
18:50 Meeting point in front of the congress center Kardinal-Döpfner-Haus

Wednesday, 1st June 2011

7. Session: Fungi

09:00 L31 ***Fusarium langsethiae* infection in wheat, barley and oat under commercial cultivation**
N. Opoku, M. Back, S.G. Edwards

09:15 L32 **Preliminary studies on the interaction between *Fusarium verticillioides* and *Fusarium graminearum* grown on different artificial media**
F.M. Ellner

09:30 L33 **Diversity of *Penicillium expansum* isolates from apples related to *idh* sequence and patulin production**
D. Colman, G. Vlaemynck, E. Van Pamel, F. Vanhove, F. Devlieghere, B. De Meulenaer, E. Van Coillie

09:45 L34 **Regulation of the alternariol biosynthesis in *Alternaria alternata***
E. Graf, M. Schmidt-Heydt, R. Geisen

10:00 L35 **Diversity and gliotoxin production of *Aspergillus fumigatus* isolates from silage: A multidisciplinary approach**
E. Van Pamel, G. Vlaemynck, M. Heyndrickx, A. Verbeken, E. Daeseleire

10:15 L36 **FUM1, NOR-1 transcript genes and mycotoxins in animal feeds from South Africa associated with animal health disorders**
P.B. Njobeh, M.F. Dutton, H. Iheanacho, E. Obiajili

10:30 Coffee break / Exhibition

8. Session: Reduction and prevention

11:00 L37 ***In vivo* efficacy testing of mycotoxin binders in poultry by means of a toxicokinetic study according to EFSA guidelines**
M. Devreese, A. Osselaere, S. De Baere, P. De Backer, S. Croubels

11:15 L38 **Influence of light on growth and mycotoxin biosynthesis of food relevant fungi**
M. Schmidt-Heydt, D. Stoll, R. Geisen

11.30 L39 **Influence of mycotoxin detoxifying agents on the plasma pharmacokinetics and tissue residues of amoxicillin and oxytetracycline in poultry**
A. Osselaere, J. Goossens, S. De Baere, S. De Saeger, M. Eckhout, P. De Backer, S. Croubels

11:45 L40 **Biological control of the phytopathogenic fungus *Fusarium culmorum* and its mycotoxin deoxynivalenol by earthworms**
F. Wolfarth, S. Schrader, E. Oldenburg, J. Weinert, J. Brunotte

12:00 Closing Session

Table of presentations

Table of lectures

Occurrence of mycotoxins

Toxicology

Analytical developments

Fungi

Reduction and prevention

Table of posters

Analytical developments

Fungi

Occurrence of mycotoxins

Reduction and prevention

Toxicology

Abstracts of lectures

Down the drain: How humans emit deoxynivalenol into surface waters

Felix E. Wettstein, Thomas D. Bucheli

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For several years, our research group has been focussing on the occurrence of mycotoxins in the aquatic environment. We were able to describe the route of zearalenone (ZON) from *Fusarium* infected plants (wheat, maize) to the soil, and into drainage water [1]. The same way of entry into the aquatic environment was investigated for the more polar and therefore better water soluble trichothecene Deoxynivalenol (DON). Indeed, DON in drainage water surpassed the values of ZON by a factor of about 200 and was also present in a majority of water samples from the river Glatt and Töss [2]. However, the question remained whether *Fusarium* spp. infected crop fields were the only source of DON. Turner et al. [3] showed that the urinary excretion of an adult U.K. citizen ranged from 6.5 to 13.2 µg DON/day, depending on the cereal intake. Applying these data with the number of inhabitants and water flow for a given waste water treatment plant (WWTP), concentrations between 20 and 50 ng/L in the influent are predicted. These numbers motivated us to investigate the fate of DON in WWTPs, including its potential input into surface waters via WWTP effluents.

The first sampling round in 2008 confirmed the occurrence of DON in WWTPs. The analyzed DON concentrations in the effluents of the primary clarifiers were within 32 and 118 ng/L. Results of the detailed study in 2009 revealed a low elimination of DON along the flow-path in WWTP Kloten/Opfikon (20% in the activated sludge tank, 15% after the sand filtration).

Conclusions

1) Our study confirms for the first time the occurrence of DON in waste water.

2) The total input of DON into a WWTP strongly correlates with the number of connected inhabitants.

3) Overall, the results of this study show that WWTP's act as another relevant input source of mycotoxins into surface waters, besides agricultural runoff.

References

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[2] Bucheli, T.D.; Wettstein, F.E.; Hartmann, N.; Erbs, M.; Vogelgsang, S.; Forrer, H.R.; Schwarzenbach, R.P., 2008. *Fusarium* Mycotoxins: Overlooked Aquatic Micropollutants? Journal of Agriculture and Food Chemistry, 56(3), 1029-1034.

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Influence of different water regimes on the deoxynivalenol concentration in *Fusarium*-infected maize kernels

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According to actual climate change predictions, air temperature and summer drought are expected to increase in great 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 weather conditions.
In order to study the consequences of changes in water availability on yield, ear fusariosis, and deoxynivalenol (DON) contamination of maize, a field experiment was conducted near Braunschweig under defined water regimes during 2009 and 2010. The supply of water was controlled with a sprinkler irrigation system installed in a mobile rain-out shelter which covered the experimental area during rainfall. The total experimental area of 162 m^2 was divided into 18 plots of equal size, where three cultivars of maize were supplied in two replicates with 50, 75, and 125% of the long-term average monthly precipitation at the experimental site. The plots were irrigated dose-related once a week during the whole cultivation period from emergence in May until harvest at mid October (2009) or late September (2010). To induce red ear rot of maize, in 2009 oat kernels infected with *Fusarium graminearum* were placed at the stem base of the plants (5 g per m row) about 6 weeks before full flowering. In 2010, the plants were inoculated at the stage of full female flowering by applying 0.5 ml of water containing 4 x 105 conidia of *F. graminearum* directly on the maize silks. At harvest, twenty ears were manually removed from plants of the two central rows in each plot. Harvested ears were dehusked and visually examined for symptoms of red ear rot. After drying the sampled ears, the kernels were separated from the rachis and ground to a fine powder (particle size: <1mm) before DON analysis by ELISA.
In 2009, the mean ear yield of the tested cultivars decreased substantially from 192 to 75 dt ha-1 (-60%), when the water supply was reduced from 125% to 50% of the long-term average monthly precipitation. The opposite trend was observed for the DON concentrations in the kernels which across cultivars increased about 5-fold from 75 to 380 µg DON kg-1 with decreasing irrigation. Concurrently with the observed weak signs of ear fusariosis in 2009, the level of DON contamination was also low in all tested cultivars.
In 2010, the mean ear yield decline from 128 to 108 dt ha-1 (-16%) resulting from decreased water supply was lower than in 2009. This can be explained with continuous natural rainfall in the surroundings of the rain-out shelter during late spring and autumn of 2010. The higher relative air humidity reduced the drought stress exerted on the maize plants. Due to more severe ear infections in 2010, the DON concentration in the kernels was at a considerably higher level. The lowest amounts of DON (cultivar mean: 330 µg kg-1) were again detected in kernels derived from the 125% water treatment, whereas about 3.5-fold higher DON concentrations were found for the 75 and 50% water treatments.
The results of this study suggest, that the risk of DON contamination of maize kernels increases with decreasing amounts of water available to the maize crop.

Impact of pre-harvest rainfall on *Fusarium* mycotoxin contamination of wheat

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The predominant species responsible for zearalenone (ZON) and deoxynivalenol (DON) production in UK wheat is *F. graminearum*. The infection and subsequent mycotoxin production by *Fusarium* spp. is largely dependent on rainfall at flowering. Matthaus et al. (2004) detected DON production post-flowering and ZON production was detected later as wheat ripened after inoculation with *F. culmorum*. Recent UK survey data has identified that ZON is more problematic than DON in seasons with delayed wet harvests. In 2008, with a prolonged wet harvest, 29% of the UK wheat crop exceeded the European Commission legal limit for unprocessed wheat intended for human consumption (100 µg/kg), whereas 13% exceeded the DON limit of 1250 µg/kg.

Mycotoxin analysis of mill fractions of wheat from a wet (2004) and dry (2005) harvest also indicated that the distribution of DON within mill fractions (bran, white flour and offal) can change depending on pre-harvest rainfall. Initial results from laboratory studies indicated that water soluble DON is readily re-distributed within the wheat grain. ZON, which is much less water soluble, appears to remain where it was produced.

Glasshouse and field experiments will be conducted to quantify the impact of rainfall between flowering and harvest on the concentration and distribution of DON and ZON in harvested wheat grains and mill fractions. Initial studies will quantify the two mycotoxins over time and relate levels detected to biomass of *F. graminearum* and expression of mycotoxin genes. Further studies will then quantify the effect of rainfall timing and duration on the concentration and distribution of DON and ZON in wheat grains at harvest and mill fractions after processing.

Reference

Matthaus, K.; Danicke, S.; Vahjen, W.; Simon, O.; Wang, J.; Valenta, H.; Meyer, K.; Strumpf, A.; Ziesenib, H.; Flachowsky, G., 2004. Progression of mycotoxin and nutrient concentrations in wheat after inoculation with *Fusarium culmorum*. Archives of Animal Nutrition, 58, 19-35.

Facts and figures: Masked *Fusarium* mycotoxins in cereals and cereal-derived products

Marthe De Boevre[1], José Diana di Mavungu[1], Aleksandrs Veršilovskis[1,2], Peter Maene[3], Kris Audenaert[4], Alfons Callebaut[2], Geert Haesaert[4], Mia Eeckhout[3], Carlos Van Peteghem[1], Sarah De Saeger[1]

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The presence of conjugated *Fusarium* mycotoxins together with their native forms is becoming a major public health concern. Therefore, the inclusion of masked metabolites in a reliable and sensitive analytical method is of utmost importance.
In this study an LC-MS/MS method was developed and validated for the simultaneous determination of deoxynivalenol, zearalenone, T2-toxin, HT2-toxin and their metabolites, including 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, deoxynivalenol-3-glucoside, alfa-zearalenol, beta-zearalenol, zearalenone-4-glucoside, alfa-zearalenol-4-glucoside, beta-zearalenol-4-glucoside and zearalenone-4-sulfate in maize, wheat, oat, cornflakes and bread.
Based on the Commission Regulation (EC) No 401/2006, the method was successfully validated for several parameters in terms of linearity, apparent recovery, limit of detection (LOD), limit of quantification (LOQ), precision, expanded measurement uncertainty and specificity. The values for LOD varied from 5 $\mu g.kg^{-1}$ to 13 $\mu g.kg^{-1}$, those for LOQ from 10 μg^{-1} to 26 $\mu g.kg^{-1}$. The results of the performance characteristics of the developed LC-MS/MS-method are in good agreement with the criteria mentioned in the Commission Regulation (EC).
During the presentation, an important matter will be raised: do masked *Fusarium* mycotoxins occur in cereals and cereal-derived products? If yes, in what quantities?
Based on the developed method, a variety of surveys was performed. Several genotypes of wheat and maize were sampled on 5 different locations in Flanders focusing on the ratio of masked mycotoxins to the total mycotoxin content. A range of masked mycotoxins from 17% to 70% was found. In addition, climatic factors, treatment strategies and soil were taken into account in order to investigate the relationship between these parameters and the amount of mycotoxins. Subsequently, ELISA (Veratox® DON High Sensitivity, Neogen Corporation) was performed on these samples and compared to the obtained LC-MS/MS results.
Quarterly per year (2010-2011), commercially available cereal based products, such as high-fiber and bran-enriched bread, popcorn, oat meal and high-fiber cornflakes were sampled and analysed. Mycotoxin contamination occurred in more than 50% of the samples. The contribution of masked mycotoxins ranged from 34% to 100%.
These results, which are the start of a three-year survey, are presented and will give a more clear view on the occurrence of masked mycotoxins versus their native precursors. This information will be used in the near future for an extended risk evaluation.

Hidden fumonisins in maize hybrids and their correlation with chemical composition

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Fumonisins are toxic metabolites produced by a number of *Fusarium* species especially in maize during the pre-harvest period, leading to risk for consumer health as well as to economical losses in particular in the Mediterranean area. An important issue posing a serious problem concerning food safety is represented by the possible presence of masked forms (hidden fumonisins) [1]. Thus, the individuation of the main factors that affect the production of fumonisins and the role played by the plant in the masking mechanism are topic of great interest in order to guarantee a good safety level for the consumer. In this study, several correlation between maize composition, maize genotype, agronomic factors and free and hidden fumonisins occurrence has been investigated in order to verify some conditions that greater affect contamination than others. At this purpose, 28 maize samples belonging to 9 different *Zea Mays* genotypes grown under different agronomical practices and characterized by different chemical composition have been collected in Northern Italy. The main agronomical parameters taken into consideration were FAO class, sowing and harvest period, preceding crop and moisture at harvest. For each sample, extractable free fumonisins and total fumonisins after hydrolysis have been determined according to our standard protocols [2] and, at the same time, humidity, total starch, protein and lipids amounts have been measured by NIR method. In order to evaluate a possible correlation between fumonisins and maize composition, a Pearson test (alpha = 0.05) was performed on the dataset, showing a positive correlation between total fat content and both free and bound fumonisins for all the considered genotypes. This correlation was better investigated by analysing the fatty acid profile, showing the same trend between linoleic acid content and free-to-total fumonisins ratio. The main hypothesis that can explain this relationship regards the role played by unsaturated fatty acids as precursors of oxylipins, which are the most important mediators in the plant-pathogen system cross talk during "in-field" infection [3]. Thus, *Fusarium* infection could encourage the biosynthesis of such compounds, leading to an increase of their intermediates. Further studies are ongoing in order to confirm these results, focusing the attention on the role played by the plant in the fumonisins masking mechanism.

References

[1] Galaverna, G.; Dall'Asta, C.; Mangia, M.; Dossena, A.; Marchelli, R., 2009. Masked mycotoxins: An emerging issue for food safety. Czech Journal of Food Science, 27, 89-92.

[2] Dall'Asta, C.; Falavigna, C.; Galaverna, G.; Dossena, A.; Marchelli, R., 2010. *In vitro* digestion assay for determination of hidden fumonisins in maize. Journal of Agricultural and Food Chemistry, 58, 12042-12047.

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Fusarin C: Forgotten over 20 years?

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Several *Fusarium* species produce the mycotoxin fusarin C, which is known to be genotoxic and immunosuppressive [1]. In addition former studies revealed a mutagenic effect of fusarin C in the Ames test after S-9 liver activation and an inhibitory effect on the production and function of macrophages [2].
In the eighties some publications demonstrated the occurrence of fusarin C in moldy corn and healthy corn kernels from the Transkei region in South Africa and in corn screenings in Pennsylvania. The co-occurrence of fusarin C with other *Fusarium* mycotoxins like moniliformin, deoxynivalenol and zearalenone was also demonstrated [3,4]. But even though this mycotoxin could be harmful to human health, only little is known about the occurrence of fusarin C in food and feed.
Therefore there is a strong need for the development of an analytical method for the detection of fusarin C in food and feed. In order to do this we had to isolate the mycotoxin since reference material is not commercially available. For the isolation *F. verticillioides* MRC 712 was cultivated in submerged culture and the culture filtrate was extracted by using solid phase extraction with C_{18}-cartridges. Final separation and purification of the fusarins were achieved using preparative HPLC.
Afterwards a protocol according to the QuEChERS-method was developed for the detection of fusarin C in food samples. The quantification is done by the use of matrix calibration and HPLC-MS/MS. To get an overview of the contamination levels of fusarin C corn kernels of maize cobs and commercially available products like popcorn maize, polenta and corn meal were examined.
Contaminated samples were also checked for their fusarin pattern, since besides fusarin C which is the most common fusarin, several structural analogs are known.

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

Mycotoxins in house dust - final results of a systematic study

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The population in countries with moderate climates spends more than 80% of their daily time indoors. About 21% of all flats in Germany have visible damage due to dampness. Approximately 10% have direct visible moulds e. g. on wallpapers, but in many cases they can be hidden behind furniture or wainscot. Particles, spores as well as semi and low volatile compounds like mycotoxins accumulate in house dust. Therefore, house dust is an ideal indicator for indoor contamination with mycotoxins. Furthermore house dust is also a possible way of exposure with toxins. More than 400 mycotoxins or metabolites from moulds are known today, but only a few reference substances are purchasable. Therefore, LC-MS offers the opportunity for sensitive and selective quantification and chemical characterization of target compounds with no need of reference substances.
In this study 500 house dust samples from the control group of a representative collection for the German population were examined. In addition 369 house dust samples from 364 dwellings were collected together with a questionnaire in different regions of Germany. 153 of the households were with and 201 without visible indoor moulds. For the investigation house dust of unknown age was collected and sieved to ≤ 63 µm particle size. The amount and species of viable moulds were determined. For the chemical analysis the mycotoxins were selected by a literature search regarding their occurrence in building materials or house dust, toxicological aspects and the availability of standards. Aflatoxin B1, Citrinin, Deoxynivalenol, Diacetoxyscirpenol, Gliotoxin, Ochratoxin A, Roridin A and Sterigmatocystin were picked up and quantified by multiple-reaction-monitoring in the tandem mass spectrometer (LC-MS/MS). Due to the fact that no macrocyclic trichothecenes from the mould *Stachybotrys chartarum* were purchasable Roridin L-2, Satratoxin G, H and their isomers were only qualitatively analysed by LC-MS/MS in the house dust extracts. Citrinin, Deoxynivalenol, Ochratoxin A, and Sterigmatocystin could be quantified in the lower µg/kg range.
The developed method is sensitive, selective and reliable for the selected target compounds. The evaluation of the questionnaires of mycotoxin-positive house dust samples compared to samples without detectable mycotoxins shows no distinct coherency to a mould infestation in the household.
Using LC-MS sensitive and selective determination of mycotoxins in house dust is possible. In addition this technique gives the opportunity for characterization and identification of mycotoxins without having reference substances. Further research is necessary to study the formation and occurrence of mycotoxins in indoor environments.

Fate of mycotoxins during milling processes

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Grain samples (wheat, rye, spelt) contaminated naturally with mycotoxins were obtained by mills across North Rhine-Westphalia. Different processes of milling/and or sorting of the mills selected may lead to different effectiveness of mycotoxin removal. Several sets of samples were analyzed applying a very sensitive and selective method of determination based on high performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS). Thus, around 30 of the most prominent toxins can be analyzed using both, negative and positive heated electrospray ionization (HESI) applying suitable internal reference standards.

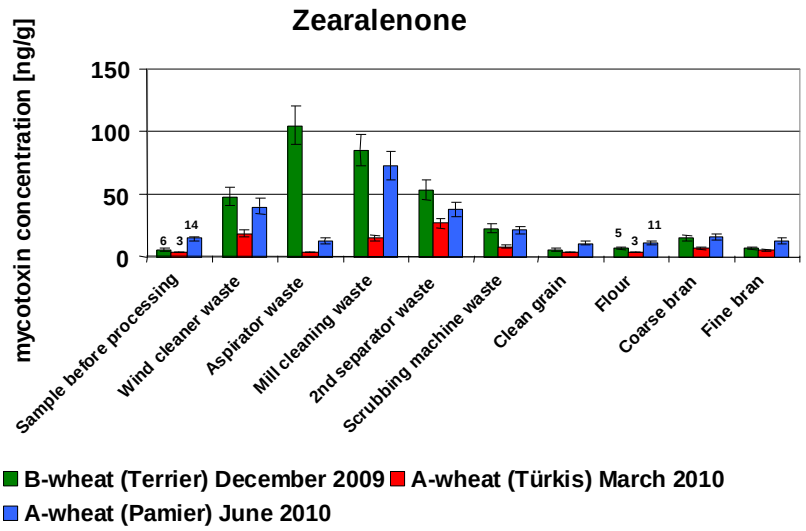

Fig.1 Behaviour of Zearalenone during milling of wheat (mill No.1)

In raw material of wheat a significant contamination with DON, zearalenone and enniatin B could be observed. Rye samples contained lower concentration of DON and higher concentration of enniatin B. As expected, colour-sorting is very effective against ergot alkaloids in rye. During milling process DON, zearalenone and enniatin B could effectively be removed from the raw material. Thus, flour showed lower concentrations.

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Occurrence of mycotoxins in beer brewed in Poland

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Mycotoxins are natural food and feed contaminants, produced by the secondary metabolism of fungi. The maximum levels of these compounds in certain food products are regulated by EU (i.e. Commission Regulation (EC) 1881/2006, 1126/2007, 105/2010, 165/2010). However, there are still foodstuffs such as beer, in which those values are considered to be set.
The possibility of mycotoxins (especially ochratoxin A – OTA) getting into beer from contaminated grains used in brewing has been pointed out in literature. On the other hand, it is considered that OTA can persist fermentation processes, but normally it can be destroyed during the malting process used in beer production.
The aim of the study was to determine OTA as well as other mycotoxins like trichothecenes and zearalenone in fifty-three Polish beer samples originating from major brewing concerns. The samples were randomly collected from various shops and supermarkets in Bydgoszcz.
The analytes were determined using HPLC with different detection modes (fluorescence – OTA, MS/MS – trichothecenes and zearalenone). OTA was isolated with the immunoaffinity columns, whereas in the case of other mycotoxins analysis, acetonitrile and celite were used during extraction and clean-up procedure. The detection and quantitation limits (OTA) were: 0.003 and 0.01 ng/ml of beer, respectively, and average recoveries ranged from 79.6% to 85.8% at different spiking levels. LODs and LOQs of other analytes varied between 0.07 – 4.3 ng/ml and 0.23 – 14.3 ng/ml of beer, respectively, and average recoveries exceeded 95%. The frequency of OTA-positive samples was 94%, while the share of samples, in which at least one of the other mycotoxins was present, was 98%.

Ergot alkaloids: Determination and occurrence in EU foods and feeds

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Ergot alkaloids are mycotoxins produced by fungi of the *Claviceps* genus, mainly by *Claviceps purpurea*. Infections are mostly prevalent in cereals and wild grasses [1]. After the infection is established, the fungus replaces the developing grain or seed with an alkaloid-containing wintering body called sclerotium or ergot. The alkaloid composition of sclerotia varies largely, depending on the maturity of the sclerotia and on other factors such as the fungal strain, the host plant, the geographical region and the prevailing weather conditions [2]. The danger caused by ergot alkaloids is well known for centuries. Although nowadays effective cleaning procedures at mills allow to remove up to 82% of ergots from grain, ergot alkaloids can still be found in feed and food commodities, sometimes at relatively high levels [1]. This presentation deals with the development and validation of a new LC-MS/MS method for the simultaneous determination of the six ergot alkaloids defined by the European Food Safety Authority (EFSA) as among the most prominent and physiologically the most active (ergometrine, ergotamine, ergosine, ergocristine, ergocryptine, and ergocornine), as well as their corresponding epimers. The experience gained through the use of the method over time with different food and feed matrices is highlighted. On the other hand, data generated from an EU survey (CFP/EFSA/CONTAM/2010/01) are presented. Commodities were sampled in 13 EU countries and include rye and wheat samples from the harvest 2009 and 2010, intended for human consumption and for animal feeding. Different cereal-based products from the Belgian food supply were also sampled. The preliminary results reveal that contamination at levels greater than 500 µg/kg is common, the feed samples being the most affected (up to 12000 µg/kg). However, there is a considerable variability in the individual and total ergot alkaloid contents. An evaluation of the individual ergot alkaloid data from the complete survey will allow to identify a marker(s) that could be used as indicator(s) for ergot contamination in food and feed.

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Acknowledgements

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Analysis of ergot alkaloids in cereal based food and feed from the 2006-2009 harvests by HPLC/FLD and LC/MSMS

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Ergot alkaloids are mycotoxins that are found in largest amounts in the fungal species of *Claviceps* genus, most notably *Claviceps purpurea*. The fungus infects the grain ears of various gramineous plants such as wheat, rye, triticale, barley, and millet and instead of sane grains large discoloured sclerotia (secal cornutum) or ergots are formed [1-4].
These sclerotia contain a variety of potentially toxic ergot alkaloids which can be classified into lysergic acid amids (e.g. ergometrine), ergopeptides (e.g. ergotamine) and iso-ergopeptides (e.g. ergotaminine). The alkaloid amount and pattern depends considerably on the producing fungus strains, the host plant, the geographical region, and the climatic conditions.
To the present, occurrence data of single ergot alkaloids in feed and food as well as information on toxicological properties are rarely published and still incomplete. Thus, the impact on humans and animals via the food and feed chain can still not be assessed adequately. In order to generate reliable data, European activities have been launched recently to monitor ergot alkaloids in food and feed [DG SANCO document 10226/2010]. Regarding this background a rapid method for the determination of selected ergot alkaloids in cereal flour was developed [5] and successfully validated in a collaborative trial for the official compilation of the German Food and Feed Code.
This method, slightly modified, was applied to 183 food and 33 feed samples from the 2007-2009 harvests. Six ergot alkaloids ergometrine, ergotamine, ergocryptine, ergocornine, ergocristine, ergosine and the corresponding D-iso-lysergic acid epimers (C-8 (S) configuration, suffix "-inine") were analysed. None of the samples exceeded the level of 1000 µg/kg, which was used as a reference level corresponding to the 0.05% maximum level of sclerotia according to the intervention regulation (EU) No 1272/2009 [6] if an average alkaloid content in sclerotia of 0.2% is applied. The results showed that HPLC/FLD analysis requires additional verification e.g. by LC/MSMS because peak identification was strongly interfered by the matrix background. Matrix effects on the overall signals of the LC/MSMS analysis were also estimated and found to be more or less negligible. A collaborative trial for method validation considering feeding stuff analysis by LC/MSMS is currently in preparation.

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Four years of wheat monitoring in Switzerland: Occurrence of different *Fusarium* species and analyses of the factors influencing the mycotoxin production

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Between 2007 and 2010, wheat samples and information on cropping measures were collected from Swiss growers. Wheat grains were examined for incidence of *Fusarium* head blight (FHB) causing species and mycotoxin content (LC-MS/MS).
From a total of 527 samples originating from 16 out of 26 cantons, three FHB causing species were dominant: *F. graminearum* (64% of all FHB species), followed by *F. poae* (20%) and *F. avenaceum* (10%). The mean content of deoxynivalenol (DON) was 650 ppb and 12% of all samples were above the European limit for unprocessed cereals (1250 ppb). The yearly mean DON content varied between 260 ppb in 2009 and 970 ppb in 2008.
The analyses of the effect of cropping measures showed that the combination of pre-crop maize and conservation tillage versus ploughing resulted in an average DON content of 2200 ppb or 370 ppb, respectively. This finding confirms results from recent on-farm experiments, which demonstrated that maize-wheat rotations with conservation tillage frequently lead to high DON contents substantially exceeding the limit (Vogelgsang et. al, in press). We also measured the content of other trichothecenes and zearalenone (ZEA). Nivalenol (NIV) and ZEA contents in samples from the same two cropping systems were lower but showed a similar pattern as those of DON (NIV: mean of 30 and 19 ppb for the two cropping systems; ZEA: 210 and 17 ppb). No correlation was found between *F. poae* incidence and the NIV content. Thus, we assume for *F. graminearum* the presence of NIV chemotypes in certain areas within Switzerland.
Chemotype investigations as well as in-depth analyses of various other cropping measures should contribute to elucidate factors that influence the occurrence and toxin contamination by the most prevalent *Fusarium* species on wheat. The hypothesis of *F. graminearum* NIV chemotypes is in line with recent observations from other European wheat surveys and presently, there are attempts to launch a concerted initiative assembling data on fungal prevalence and toxins from various geographic areas in order to establish a European map on FHB chemotypes.

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Monitoring of Swiss commercial grain maize: Occurrence of *Fusarium* species, mycotoxins and the effect of cropping factors

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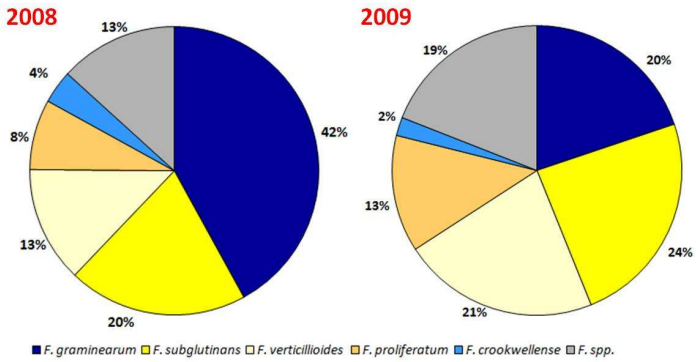
Maize is frequently attacked by *Fusarium* species, causing ear and stem rot. Infection may lead to severe yield losses and contamination with mycotoxins, threatening human and animal health. Based on a 3-year monitoring of Swiss commercial grain maize samples, the *Fusarium* species composition, the mycotoxin contamination and the potential influence of different cropping factors were investigated. The aim was to assess the impact of *Fusarium* species in maize production and to develop recommendations for the growers to reduce the risk of infections and to contribute to safe food and feed.
A total of 293 maize grain samples out of 14 cantons were evaluated by using the agar plate method to determine the *Fusarium* species incidence [1]. Deoxynivalenol (DON), zearalenone (ZEA) and fumonisin (FUM) concentrations were determined with an ELISA test. With 15 different *Fusarium* species isolated, we found a high species diversity. Overall *Fusarium graminearum* (FG) *F. subglutinans*, *F. verticillioides* and *F. proliferatum* were the most dominant species. But their incidence varied between the years (Fig. 1; samples of 2010 still in process).



Fusarium species composition and frequency from maize kernels in 2008 and 2009.

In 2008, FG was clearly dominant, whereas in 2009, the four mentioned species occurred in equal parts. A similar year effect was also observed for the level of DON-contamination: In 2008, about 40% of the grain maize samples exceeded the regulatory maximum limit of 1.75 ppm (raw maize for human consumption), whereas in 2009 and 2010, 10% and 48% of the samples exceeded this limit, respectively. In all three years, low concentrations of ZEA and FUM were measured. Hence, concerning the influence of different cropping factors, we concentrated on FG and DON. Based on the data from 2008 and 2009, only trends could be observed. Maize grain samples from fields with small-grain cereals as the pre-crop and with reduced tillage, showed a higher rate of FG infestation and higher DON contents. Our monitoring revealed a high impact of *Fusarium* species in Swiss maize production. Based on the high proportion of FUM producing species, the potential risk for FUM contamination could be considerably higher than observed in this study. The results demonstrate a great need for improved cropping systems and low susceptible maize cultivars.

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New aspects regarding deoxynivalenol in chicken

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Deoxynivalenol (DON) is a well known undesirable, immunosuppressive and highly uneconomic mycotoxin which commonly contaminates feed ingested by husbandry animals.

Our broiler DON feeding trials and investigations revealed that DON concentrations in broilers gut content are varied and metabolised within the small and large intestine. Bacterial population shifts in the gut may play an important role in this regard. The immune system within and outside the gut wall indicates a crucial part in undulating serum titer levels against viral infectious bronchitis, Newcastle disease and viral bursal disease. Heterophilic granulocytes and lymphocytes are influenced indicating stress and can get easily registered in the blood picture of broilers.
Chronic or lifetime exposition of low and high DON, natural *Fusarium graminearum* culture extract enriched with DON and other mycotoxins in broilers' feed alter gut macroscopic and microscopic morphology before any performance reduction can be measured. The gut wall, gut content and microbiota, and gut connected immune systems of broilers are primarily the first targets of ingested DON contaminated feed. Reactions of inner organs (spleen) and systems such as gut, microbiota and immune system are counteracting the unwanted DON in different, however, measurable levels. Damage of the epithelium of jejunum in terms of lower and broadened villi are measured. DON and DON-metabolite enrichment is determined in cecum content. Single oral DON application results in lower DON plasma concentrations in birds reared on high DON diets.
Low protein (half the recommendation) in the grower phase feeding of broilers in combination with DON results in significant lymphocyte DNA fragmentation.
The most important pathohistological findings, vaccination titers, blood and organometric variables are shown during the presentation of our results.

The conclusion of the DON fed broiler trials is that DON alone and/or in combination with other unwanted feed ingredients targets complex gut organs, inducing vice versa time and dose dependent counter and compensation reactions of the gut and connected systems. These changes can be observed earlier before any impact on zootechnical performance such as feed intake, weight gain, and feed conversion.

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Effect of deoxynivalenol (DON) on health and performance of laying hens of different genetic background

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Deoxynivalenol (DON, vomitoxin) is a well known type B trichothecene and one of the most often detected mycotoxins in cereals and feed stuffs. The sensitivity to DON varies between animal species. As poultry is regarded to be less sensitive to DON it seems possible that contaminated cereal batches are diverted to poultry feed, resulting in an increased exposure of this animal category. In addition it seems conceivable that laying hens from a different genetic background may react differently to the presence of DON in feed. Therefore the aim of the present study was to investigate the effects of graded levels of DON in diets for laying hens of different genetics.

Methods: Three diets with increasing concentrations of DON (0-5-15 mg/kg) were investigated in a feeding experiment with 216 laying hens (23 weeks old) for 12 laying month. Half of the animals were Lohmann Brown (LB) and Lohmann Silver (LSL), respectively, resulting in six experimental groups of 36 hens each. Feed and water were supplied for *ad libitum* consumption. The amount of feed consumed was recorded weekly while laid eggs were counted daily. At the end of the experiment 20 laying hens per group were slaughtered. Liver, spleen, heart, glandular stomach and gizzard were dissected, emptied, weighed and prepared for further analysis. The data were evaluated by ANOVA; means were compared by Fischer-LSD test ($p<0.05$).

Results: Significant interactions between DON, breed and laying month were observed on feed intake, daily egg mass and laying intensity (Figure 1). In general the performance was decreased by the presence of DON in feed within the twelve laying months but to a different extent and depending on the breed. However, no significant effects were observed on feed conversion. Moreover, significant interactions between DON and breed were observed on the relative weight of liver and breast muscle. Body weight and relative gizzard weight (Figure 2) were significantly decreased by the presence of DON in feed. The other relative organs weights were not influenced by dietary treatments.

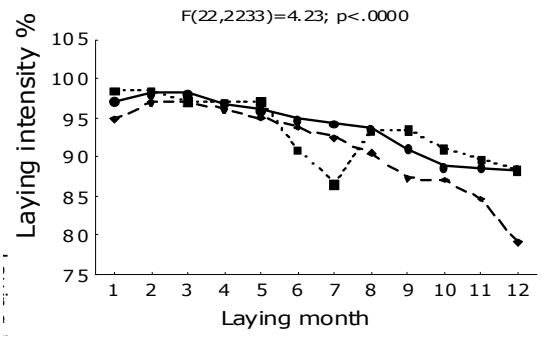

Figure (1): Effect of increasing concentrations DON in feed on laying intensity within the 12 laying months.

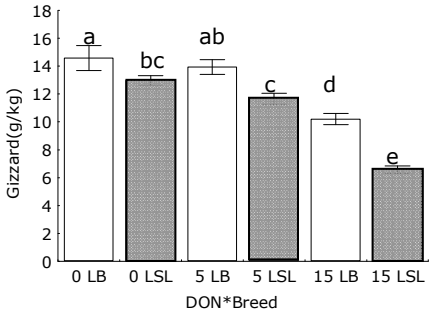

Figure (2): Relative weight of gizzard in the experimental groups (means ± SE, n=20). a-e different Letters show significant differences, ($p<0.05$).

Conclusion: Under the conditions of this study laying hens of different genetic backgrounds appear to have different sensitive reactions to the presence of DON in feed. However, additional studies should be conducted to clarify these results.

Immunotoxicological effects in progeny of hens fed aflatoxin and ochratoxin contaminated rations

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The objectives of the present study were to determine the immunotoxicological effects in the progeny of breeder hens fed rations contaminated with either ochratoxin (OT), aflatoxin (AF) or their combination.

White Leghorn breeder hens of 40 weeks of age kept in six groups were fed rations contaminated with OTA (3.0 and 5.0 mg/kg) and AFB1 (5.0 mg/kg) alone or concurrently for 3 weeks. The hens were artificially inseminated with the semen obtained from the cocks of same breed kept on basal feed. OTA and AFB1 residue were detected in eggs, liver, kidneys and muscles. Concentrations of residues of OTA and AFB1 in tissues of the groups fed both mycotoxins concurrently were significantly lower than the groups fed AF and OT alone. Effect of both mycotoxins fed alone or concurrently had inconsistent effect upon fertility of the eggs. An increase in embryonic mortality and decrease in hatchability was observed in groups fed OT or AF. These changes were more severe in groups fed both the mycotoxins concurrently. Progeny obtained from OT and AF fed hens had significantly lower body weights than controls. Relative weights of liver and kidneys were higher in the progenies of hens fed toxins contaminated feed. Relative weights of bursa of Fabricius and spleen were significantly lower in progeny of hens fed aflatoxins and ochratoxins. An increase in relative weight of liver and decreased in that of bursa of Fabricius was significantly more severe in progeny from hens fed both AF and OT concurrently than those fed these toxins alone. Lymphoblastgenic response to PHA-P determined by skin thickness and *in vivo* phagocytic potential of circulatory macrophages determined by carbon clearance assay were more severely depressed in the progenies obtained from the hens fed OT and AF concurrently than the groups fed these mycotoxins alone. Immunoglobulin containing cells in the spleen and bursa of Fabricius were significantly lower in the progenies of hens fed AF and OT concurrently than the groups fed these toxins alone. Peritoneal macrophages following challenged with LPS showed a decreased phagocytosis of sheep red blood cells (SRBC) and low nitrite production. These decreases in these responses were more severe in the progenies from the hens fed both the toxins concurrently. Antibody titers to SRBCs were significantly lower than control group in progenies of hens fed both toxins concurrently.

Use of different biomarkers to evaluate the toxicity of hydrolyzed fumonisin B1

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Introduction: Fumonisin B1 (FB1) is a cancer promoting mycotoxin. As recently shown [1] the enzyme FumD is capable of removing both tricarballylic side chains, resulting in hydrolyzed FB1 (HFB1). The aim of this work was to investigate if this conversion may be regarded as detoxification. To this end, the effects of HFB1 on the sphingolipid metabolism should be investigated and the concentrations of HFB1 in tissue, plasma and faeces samples of piglets receiving HFB1 should be determined.
Experimental: Three groups of 6 piglets each received 2 mg/kg BW FB1 (FB1-group), the equimolar amount of 1.12 mg/kg BW HFB1 (HFB1-group) or buffer solution (control group) by gavage daily for the duration of 2 weeks. Plasma and faeces samples were taken after 0, 7 and 14 days, tissue samples were obtained after euthanasia at the end of the experiment. Plasma and tissue samples were investigated for the sphinganine to sphingosine ratio by HPLC-FLD and all samples were analyzed for the concentrations of FB1, partially hydrolyzed FB1 and HFB1 by LC-MS.
Results and discussion: Elevation of the sphinganine to sphingosine (Sa/So) ratio in tissues and plasma is a biomarker for consumption of FB1. Whereas the average Sa/So ratios were greatly elevated in the FB1-group [2], the average Sa/So ratios of the HFB1-group were not significantly increased. However, in two of the six piglets of the HFB1-group the Sa/So ratios in lung and liver samples increased to 0.45-0.55 compared to 0.11-0.15 in the control group. These pigs also exhibited increased concentrations of HFB1 in lung samples (4.1-5.3 µg/g). In lung samples of the other pigs of the HFB1-group, HFB1 concentrations ranged from 0.005 to 0.64 µg/g. In other tissues of the HFB1-group, HFB1 concentrations were below 0.005 µg/g and with that in the range of FB1 concentrations in all tissues of piglets of the FB1-group. The average FB1 concentration in freeze dried faeces was 103 ± 37 µg/g, the average HFB1 concentration reached 66 ± 43 µg/g.
Conclusion: Contrary to administration of 2 mg/kg BW FB1, dosage of the equimolar amount of HFB1 to growing piglets by gavage had only minor influence on the sphingolipid metabolism. Tissue concentrations of FB1 and HFB1 were low with few exceptions. Great differences in the HFB1 concentrations in lung were observed in the HFB1-group. A positive correlation between elevated Sa/So ratios and increased HFB1 concentrations in lung was observed for piglets of the HFB1-group.

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New fumonisin metabolites: *In vivo* formation of N-acyl-fumonisin B1 by the ceramide synthase

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The interaction with the sphingolipid metabolic pathway based on the inhibition of the key enzyme, ceramide synthase, is supposed to be the cytotoxic mode of action of the fumonisin mycotoxins. In order to investigate new details concerning the interaction of the predominant fumonisin B1 (FB1) with the enzyme ceramide synthase (CerS) and to answer the question whether fumonisin based analogs of ceramides are formed, we employed a combination of biochemical methods and instrumental analytical techniques. Furthermore we analyzed the interaction of FB1 with the six overexpressed mammalian isoforms of the ceramide synthase (CerS), resulting in complex modifications of the sphingolipid metabolism. Due to the synthesis of standard compounds and the development of a highly sensitive HPLC-ESI-MS/MS method we report for the first time the formation of N-acyl-FB1 with various fatty acid chain lengths by the mammalian enzyme ceramide synthase. Furthermore we investigated the substrate specifity of six mammalian CerS isoforms regarding FB1 and HFB1. Therefore we applied transient overexpression of the CerS isoforms genes in HEK293T cells to investigate the substrate specifity concerning the N-acylation of fumonisin B1. The comparison of the N-acyl formation of fumonisins with their hydrolyzed derivatives indicates that the specific structural differences are crucial for the biotransformation by ceramide synthase isoforms *in vivo*. The biosynthesis of specific complex sphingolipids was inhibited by these new fumonisin metabolites, which was analyzed by the quantification of a broad range of sphingolipids in CerS-overexpressing cell lines. Furthermore the N-acyl-FB1-deriatives are cytotoxic in human primary cells, with the strongest effect for the short chain derivatives.

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Trypacidin, a spore borne toxin from *Aspergillus fumigatus*, induces cytotoxicity in lung cells

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Aspergillus fumigatus is a saprophytic fungus that can grow outdoor on different organic materials but also indoor especially on damp wallpapers [1]. Inhalation of *A. fumigatus* conidia can cause severe aspergillosis in immunodepressed people [2]. A large number of secondary metabolites are possibly produced by *A. fumigatus*, depending on the growth conditions of the fungus, but only a limited number are airborne by the spores [3] whose toxic effect to the respiratory tractus had not been investigated.
We found that spores of *A. fumigatus* bear five main compounds, tryptoquivaline F, fumiquinazoline C, questin, monomethylsulochrin and trypacidin. Fractionation of culture extract using RP-HPLC and LC-MS analysis showed that samples containing questin, monomethylsulochrin and trypacidin caused toxic effect to the human A549 lung cell line. These compounds were purified and their structure verified using NMR in order to compare their toxicity on A549 cells. Trypacidin displayed the most toxic effects, decreasing the cell viability and triggering cell lyses, both effects occurring at an IC50 close to 7 µM. Trypacidin toxicity has also been observed in the same concentration range on human bronchial epithelial cells. Trypacidin initiates very early a cell death process involving the intracellular formation of nitric oxide (NO) and hydrogen peroxide (H_2O_2): indeed only one hour exposure was efficient to trigger a cell death within the following 24 hours. This process leads to a necrotic cell death as shown by the cell lyses. Moreover, trypacidin did neither induce cell cycle arrest nor apoptotic bodies nor decrease of the mitochondrial membrane potential confirming that the apoptosis pathway is not involved in the cell death process.
Since the identification of trypacidin and the demonstration of its anti-protozoal properties in 1963 [4], it is the first time that the toxic property of trypacidin for lung cells is reported, suggesting its potent role in the physiopathology of Aspergillosis.

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Combined effects of deoxynivalenol and *Salmonella* Typhimurium on intestinal inflammation in the pig

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Deoxynivalenol (DON) is a trichothecene mycotoxin which frequently contaminates maize and small grain cereals in European countries with a temperate climate [1]. Pigs are considered particularly sensitive to the dietary intake of DON with as a major consequence substantial economic losses [2]. *Salmonella* Typhimurium is the predominant serovar isolated from pigs causing also economic losses and posing serious public health problems as pigs serve as a reservoir of infection for humans [3].
The intestinal epithelial cells play an important role as a dynamic barrier, but also take part in the immune system by producing cytokines and chemokines crucial for recruitment and activation of several immune cells [4]. Ingestion of DON through contaminated feed may lead to high concentrations of the toxin in the intestine resulting in both barrier disruption and stimulation of the production of inflammatory cytokines by the intestinal cells [5,6].
A ligated intestinal loop model was used to evaluate whether the presence of relevant concentrations of DON could exacerbate the intestinal inflammation induced by *Salmonella* Typhimurium infection. In two 5-week-old piglets twelve loops were created in the ileum and 4 test conditions were randomly assigned: 1) negative control (Luria-broth, LB), 2) 1 µg/mL DON in LB, 3) 4 x 108 cfu/mL of *Salmonella* Typhimurium, 4) 4 x 108 cfu/mL of *Salmonella* Typhimurium in combination with 1 µg/mL DON. Six hours post administration, the pigs were euthanized and the loops were excised and processed for RNA isolation. To quantify the cytokines and chemokines of interest (IL-1beta, IL-6, IL-8, IL-12, IL-18, TNFalpha, IFNgamma and MCP-1) real-time quantitative PCR was utilized.
Results revealed that cotreatment with 1 µg/mL of DON potentiates the intestinal cytokine and chemokine response to *Salmonella* Typhimurium in ileal tissue by means of a higher mRNA expression of IL-1beta, IL-12, IL-8, MCP-1, TNFalpha and IL-6, indicating an enhancement of the intestinal inflammation which could be harmful for both animal and human health.

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Mycotoxin related cytotoxicity of grain products and production waste from grain mills in North-Rhine Westphalia

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In a joint project carried out in North-Rhine Westphalia, Germany, typical products from grain mills were analysed by LC-MS/MS for mycotoxin content by a project partner (see Zühlke et al., this volume). Analysis were carried out with wheat, rye and spelt from conventional and organic farming, and included grains, flour, wholemeal flour and bran, as well as waste fractions from the milling process. Extracts of known mycotoxin composition were then used to determine cytotoxicity in V79 cell cultures by means of the neutral red assay (Föllmann et al. 2009, Mycotoxin Research, 25, 1-19). Mycotoxins, in concentrations comparable to the identified levels in the samples, were studied in parallel as references (for the predominant component and mixtures).
Extracts from flour and wholemeal flour samples (for human consumption) induced no measurable cytotoxicity, a result in line with the low levels of deoxynivalenol and enniatin B (from not detectable up to 0.4 µg/g DON and 0.5 µg/g EnnB) in these samples. In contrast, bran samples were cytotoxic although their level of mycotoxin contamination was also low. Cytotoxicity of bran samples from wheat was lower compared to bran samples from rye and spelt. The cytotoxic effects of the bran samples cannot be related to their mycotoxin content since the pure mycotoxins and combinations tested in parallel showed no cytotoxicity at the concentrations present in the product.
Waste materials from different stages of the milling process were often contaminated with several mycotoxins: Enniatin B was detectable in nearly all samples, but also T-2 toxin, HT-2 toxin, ergotamine, ergocornin and deoxy-nivalenol were found. Waste sample extracts with notable mycotoxin levels (up to 6 µg/g DON, 7 µg/g EnnB, 16 µg/g ergotamine, 50 ng/g HT-2 toxin) exerted pronounced cytotoxicity in cells, clearly stronger than expected based on cytotoxicity assays with mixtures of reference mycotoxins tested in parallel. Presently we investigate whether this is due to overadditive effects of mycotoxin mixtures and/or related to other cytotoxic contaminants in the real samples.
In summary, flour and wholemeal flour extracts contained relative low levels of mycotoxins and were not cytotoxic. In the case of bran we observed cytotoxicity in extracts which cannot be explained by the mycotoxin content. This phenomenon and combination effects require further studies.

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Functional genomic approach to examine the effects of *Fusarium* toxins on the immune response of pig: DNA microarray expression profiling in ileum/Peyer's patches

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Mycotoxins are toxic metabolites of moulds harmful to humans and animals. In particular, mycotoxin deoxynivalenol (DON) is prevalent worldwide in crops used for food and feed production. Furthermore, DON is resistant to milling, processing and heating, and it can easily enter human and animal food chains.
Among animals, pigs show the greatest sensitivity to DON. Consequently, DON is considered to be the major cause of economic losses in pigs that are worldwide important food source. In addition, pig is an important model organism for biomedical research for human. Ingestion of DON significantly affects the global and the specific immune response of the pigs. However, it can act either immunostimulatory or immunosuppressive depending on dose, frequency and duration of exposure. Therefore, DON can alter immune response after vaccination and modulate lymphocyte response [1, 2].
Thus, the goal of this study was to elucidate the effects of 3 days exposure to naturally occurring doses of *Fusarium* toxins on intestinal immune response of pig. For this purpose pigs were fed a control diet (DON 0.4 mg/kg, 3-ADON<0.05 mg/kg, 15-ADON<0.10 mg/kg, zearalenone 0.02 mg/kg) and contaminated diet (DON 4.7 mg/kg, 3-ADON<0.05 mg/kg, 15-ADON<0.05 mg/kg, zearalenone 0.5 mg/kg). There was no difference in feed consumption between the groups. After 3 days, the terminal portion of the ileum enriched with Peyer's patches from each animal were collected, flash-frozen in liquid nitrogen and stored at -80°C. Affymetrix microarrays were used to assess the effects of DON on ileum/Peyer's patches transcriptome. Microarray data were validated by quantitative real time PCR.
The microarray findings will be compared between the groups and discussed. Results of this research can yield new insights into the actions of DON on immune response in pig as well as in human. There is a need to better understand the mechanist linkage between these early dose-dependent molecular effects and relevant pathological consequences to develop appropriate protective approaches [3]. Results of such study can result in potential new markers of DON exposure with potentials to enable epidemiological studies to refine risk assessment and regulatory standards for this mycotoxin.

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Effects of increasing concentrations of deoxynivalenol, zearalenone and ochratoxin A in diets for Atlantic salmon (*Salmo Salar*) on performance, health and toxin residues

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The incorporation of cereal grains into diets for carnivourous fish, like Atlantic salmon, aims to improve sustainability of fish farming as marine resources of protein and lipids are limited and associated with ecological and ethical concerns. However, the use of grain in diets for fish may also introduce contaminants typically associated with those feed stuffs, such as mycotoxins. Little information on the effects of frequently occurring mycotoxins, like deoxynivalenol (DON), zearalenone (ZON) or ochratoxin A (OTA), on performance and health of salmon are available. Thus, no evidence based recommendations on maximum concentrations of those toxins in feed for salmon exist. A general recommend-dation on guidance values of DON in complementary and complete feedingstuffs of 5 mg/kg (at 88% dry matter) was given by the European Commission (2006/576/EC). Concerning ZON and OTA only guidance values for feed materials of 2 mg ZON/kg and 0.25 mg OTA/kg exist. Therefore a feeding trial was carried out to investigate the effects of those mycotoxins on the performance and health of Atlantic salmon and on toxin residues in fish tissue.
A total of 36 tanks (1 m diameter) with 22 fish each were distributed to 12 feeding groups (3 tanks/diet). Dietary treatments included control group, containing background levels of mycotoxins (0.3 mg DON, 0.03 mg ZON, 0.0 mg OTA/kg), 4 increasing concentrations of DON (0.8 – 3.7 mg/kg), ZON (0.06 – 0.77 mg/kg), and 3 increasing concentrations of OTA (0.09 – 0.33 mg/kg). Animals had a mean initial weight of 405 ± 31 g and were fed *ad libitum* for 15 weeks. At the end of the experimental period 10 fish per tank were sampled for blood plasma for clinical chemistry. From 3 fish per tank organ weights were recorded and tissue samples were prepared for histological examinations. Mycotoxin residues were analysed in plasma and muscle according to the method presented by Valenta et al. (2011).
Feeding the diet containing 3.7 mg DON/kg resulted in a significant decrease in feed intake of 20% as compared to control. As the feed conversion ratio in this group was significantly increased by 18% the effects amounted to a 31% decrease of the specific growth rate. In addition, DON-dose dependant alterations of plasma enzymes hint at adverse effects on the liver, which, however, were not reflected by the histological evaluation. Feeding diets containing increasing concentrations of ZON or OTA did not affect feed intake or growth. The con-centrations of DON in plasma and muscle of the fish increased linearly corresponding to the concentrations in the diets, while the metabolite de-epoxy-DON was not detected. ZON, OTA and metabolites of those mycotoxins could only be detected in trace amounts in samples of single fish. The results of this experiment demonstrate that current recommendations on DON in feed do not prevent adverse effects on performance and health of Atlantic salmon.

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Development and application of a stable isotope dilution assay for tenuazonic acid

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Introduction: Tenuazonic acid (TA) is a mycotoxin predominantly produced by several species of the black mold *Alternaria*. However, *Piricularia orycae*, *Phoma sorghina* and even *Aspergillus* were reported to produce this substance as well. TA belongs to the group of 3-acetyl-tetramic acids that exhibit not only strong acidic but also metal chelating properties. Therefore, determination of TA with reversed phase high performance liquid chromatography requires addition of Zn^{2+} or EDTA (ethylenediaminetetraacetic acid) to the mobile phase to ensure reproducible chromatographic behavior. As this approach is not compatible with liquid chromatography tandem mass spectrometry (LC-MS/MS), a derivatization method using 2,4-dinitrophenylhydrazine (DNPH) was recently reported [1]. Nevertheless, it is well known that derivatization reactions have to be thoroughly optimized in terms of concentration of the derivatization reagent and derivatization time for every different matrix to ensure complete derivatization of the analyte. It has been shown that the use of stable isotope labeled standards compensates for unsteady derivatization [2]. Consequently, it was the aim of this work to synthesize stable isotope labeled TA and to develop a stable isotope dilution assay (SIDA) for TA based on derivatization with DNPH.

Results and Discussion: [$^{13}C_6$,^{15}N]-TA was synthesized in a micro-scale approach from [$^{13}C_6$,^{15}N]-isoleucine by Dieckmann intramolecular cyclization after acetoacetylation with diketene with an overall yield of 30% [3]. The synthesized [$^{13}C_6$,^{15}N]-TA was used as internal standard for the determination of TA in food commodities by LC-MS/MS after derivatization with DNPH [2]. The developed SIDA was validated for different food matrices. The limits of detection were 0.15 µg/kg (juices and tomato products), 1.0 µg/kg (cereals) and 17 µg/kg (spices). The limits of quantitation were 0.5, 3 and 50 µg/kg, respectively. The recovery from all matrices was close to 100% in the range of 3 – 30000 µg/kg. Inter-assay precision was determined by repetitive analysis of naturally contaminated samples (juices, tomato ketchup, cereals and spices). The relative standard deviation was below 5% in all cases. TA occurred frequently in a series of samples, with tomatoes and spices being the most contaminated food commodities.

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Molecularly Imprinted Polymers (MIPs) towards twelve ergot alkaloids: Application in the clean-up of samples prior to LC-MS/MS analysis

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Ergot alkaloids are mycotoxins produced by fungi of the *Claviceps* species, specifically *C. purpurea,* which can be found on cereals. These fungi produce sclerotia during the growth of cereals and these sclerotia vary in composition and concentration of ergot alkaloids. They are harvested together with the grains, thus posing a potential threat toward human and animal health. Only up to 80% of the sclerotia can be removed with the mechanical separation techniques currently available. An excess intake of ergot alkaloids can cause nausea, convulsions, hallucinations and even gangrenous symptoms in humans. Weight loss, raised death ratios and other adverse effects have been reported in livestock leading to substantial economic losses.

MIPs towards the twelve ergot alkaloids described as being the most important ones in the EFSA opinion of 2005 - ergocristine, ergotamine, ergocryptine, ergometrine, ergosine, ergocornine and their corresponding epimers - have been developed. Metergoline, an ergot alkaloid structural analogue, was used as template. The MIPs were obtained through suspension polymerisation rendering beads with a narrow size distribution and an average particle size of 30 µm.

The MIPs were applied as a sorbent in solid phase extraction columns used in the clean-up of barley samples. These samples were submitted to a molecularly imprinted solid phase extraction (MISPE) procedure and optimisation of the subsequent steps of this procedure led to the elution of the desired ergot alkaloids. Recoveries for the MIPs between 65% and 79%, as well as acceptable relative standard deviations, were obtained for all compounds. Inter-day precision was tested for different batches of MIPs and the recoveries for all compounds were situated between 56% and 71%, clearly differing from the NIP recoveries with values between 19% and 23%. A cross-reactivity study was performed with a wide range of mycotoxins such as nivalenol, fumonisins, aflatoxins, etc.

The entire procedure of extraction and MISPE was evaluated through determination of the apparent recoveries. These values were situated between 95% and 119% for all compounds except ergocryptine and ergocryptinine. The lowest quantities measured were between 1 – 10 µg/kg, however lower detection limits might be established.

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Advanced synthesis of masked mycotoxins - methods and applications

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During phase II detoxification in plants, animals or humans xenobiotic substances like mycotoxins are conjugated to a hydrophilic moiety, for example glucose, glucuronic acid or sulfate. These conjugates, the so called masked mycotoxins, are usually not detected using standard protocols and quantification is not possible due to lack of reference materials. Therefore this class of substances is getting more and more in the focus of synthetic chemists.
Compared to the well studied synthesis of oligosaccharides the preparation of mycotoxin glucosides and glucuronides is more difficult due to stability problems of these target molecules during deprotection in the last step. Classic esters (e.g. acetyl or benzoyl) are commonly used as participating protective groups in carbohydrate chemistry, but deprotection under basic conditions is not compatible with ester or acyloin functionalities as it is the case in zearalenone (ZON) or deoxynivalenol (DON) respectively.
Applying classic methods for the synthesis of ZON4GlcA led to stability problems during final deprotection even after optimization of reaction conditions. Therefore we decided to avoid this problem by using new strategies and methods for the synthesis of other masked mycotoxins.
New glycosyl donors (for glucosylation, malonylglucosylation and glucuronidation) were synthesized using different protective group patterns and different leaving groups at the anomeric centre (fig. 1).

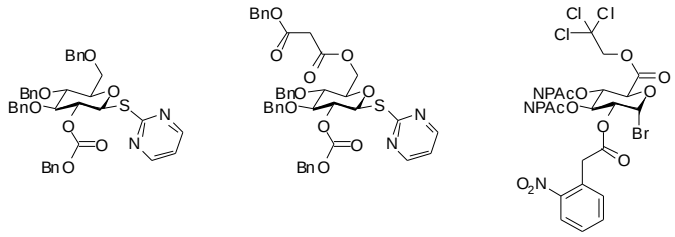

Figure 1: Examples of new glycosyl donors (Bn = benzyl, NPAc = (2-nitrophenyl)acetyl)

Benzyl ethers as well as benzyl carbonates can be cleaved using catalytic hydrogenation and (2-nitrophenyl)acetyl (NPAc) as well as 2,2,2-trichloroethyl groups are deprotected under reductive conditions.
Methods for the synthesis of mycotoxin sulfates (e.g. AOH3S and ZON4S) were also developed and optimized.
We are now able to present these reliable procedures for the synthesis of masked mycotoxins as well as the first applications.

Ochratoxin A metabolites in human blood and urine

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Ochratoxin A (OTA) is nephrotoxic in all animal species tested, and has been classified as potential human carcinogen by IARC (Group 2B). Presence of OTA in blood, urine and/or milk is indicative of frequent human exposure to the mycotoxin [1,2]. Biomonitoring studies so far have measured parent compound in blood and in urine, and thus data on OTA-metabolites in humans is scarce or contradictory [3,4]. In this work, OTA and its metabolites (OTα, 4*R*-OH-OTA and phase II metabolites) were assessed in blood plasma and urine with the aim to further improve biomonitoring methods for this mycotoxin.
A small cohort of 16 healthy adults was asked to provide spot urine and blood samples on the same day. Samples were either directly extracted or treated with ß-glucuronidase/arylsulfatase (enzymatic hydrolysis) prior to extraction with chloroform/isopropanol [5]. Analysis and quantification of samples with isotope labelled internal standards (OTA and OTα) was done by HPLC - MS/MS.
All plasma samples were positive for OTA, the major form in blood; in a range of 100 – 1600 ng/L. Enzymatic hydrolysis did not increase OTA levels, indicative of the absence of OTA-conjugates. The stereoisomer of OTA (14*R*-OTA) was also frequently detected, but at lower levels. The hydroxylated 4R-OH-OTA and the non toxic metabolite OTα were rarely detected and appear to be minor OTA-metabolites in blood samples of this cohort.
Urine samples were also positive for OTA aglycone; however, the concentrations were up to 10 times lower than those in blood plasma. Interestingly, the urinary OTA levels increased after enzymatic hydrolysis (up to 300 ng/L). 4*R*-OH-OTA was detectable in less than 50% of the urine samples and can be present as conjugate. In contrast to previous studies where OTα represented the major component in human urines [5], this metabolite was a minor constituent in urine samples from this cohort.
In conclusion, a comparison of OTA levels and metabolite patterns in blood and urine samples from several volunteers revealed interesting differences in OTA metabolism. Further biomonitoring studies for OTA should include an analysis of phase-I and phase-II metabolites.

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UHPLC-MS/MS based method for the determination of mycotoxins regulated in the European Union using stable isotope labelled standards

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LC-MS based multi-target methods for the simultaneous determination of mycotoxins in different commodities are steadily increasing in popularity. However, accurate mass spectrometric quantification is hampered by matrix effects caused by suppression or enhancement of the analyte signal. Possible approaches to cope with matrix effects are dilution of the sample, matrix matched calibration, standard addition or internal calibration. The latter approach is promising because fully ^{13}C-labelled internal standards (ISTD) are available for all mycotoxins regulated by European Commission Regulation (EC) No 1881/2006 and its amendments.

Here we present a novel highly accurate UHPLC-MS/MS based method for the quantification of regulated mycotoxins. The method includes the determination of aflatoxins (AFB_1, AFB_2, AFG_1, AFG_2), fumonisins (FB_1, FB_2), ochratoxin A (OTA), deoxynivalenol (DON) and zearalenone (ZON), as well as T-2 and HT-2 toxin. For the latter two mycotoxins no maximum residue levels (MRL) are set at the moment, but are expected to be implemented into European legislation in the near future. A multiple extraction with acidified acetonitrile-water mixtures was performed at room temperature on a rotary shaker. After extraction an aliquot of the raw extract was spiked with 11 fully ^{13}C-labelled internal standards and dried down by a gentle stream of nitrogen. For up-concentration, the residue was dissolved in a smaller volume with an acetonitrile-water mixture and 5 µL were directly injected into the UHPLC-MS/MS system without further clean-up. Analysis was performed in the multiple reaction monitoring (MRM) mode using electrospray ionization (ESI) and fast polarity switching. Method performance characteristics were determined by spiking blank samples of model matrices with native mycotoxins before extraction on multiple levels in triplicate. Furthermore, the linear range was determined and the limit of detection (LOD) was estimated at a signal-to-noise ratio of 3:1.

The use of an UHPLC-MS/MS system improved the chromatographic resolution and the multiple extraction steps enhanced the extraction efficiency of the target analytes. The application of fast polarity switching allowed the determination of the mycotoxins in the most abundant ionization mode in one chromatographic run. The addition of internal standards to the raw extract compensated matrix effects and minimized the costs. The linear range covers 2 to 3 orders of magnitude and LODs were in the low µg kg-1 range.

New insights into multi-mycotoxin analysis by LC-MS/MS

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In the recent years, LC-MS/MS based multi-analyte methodology has been successfully introduced into the field of mycotoxin analysis. Most of the related methods, however, still focus on those mycotoxins that are addressed by regulations as well as on some of their derivatives, which makes up approximately two dozens of compounds. This is partially due to the fact that many researches still rely on some sort of sample clean-up (e.g. a QUECHERS-like approach [1]), which limits the number of analytes as some of the target substances might not be amenable to the chosen procedure. However, even most of the "dilute-and-shoot" approaches seem to focus rather on improving the limits of detection for a few target mycotoxins (see e.g. [2]) than on including a broader range of target analytes.
One aim of this presentation is to show that the analytical performance of the latest generation of tandem mass spectrometers is not compromised by the simultaneous determination of hundreds of analytes. Based on selected examples (e.g. occurrence of *Alternaria* and "emerging" *Fusarium* metabolites in grains, pattern of fungal metabolites in silage samples) the merits of such a multi-analyte approach in the field mycotoxicology will be discussed.

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C8 -2'- deoxyguanosine OTA-adducts detection and OTA metabolites in biologic fluids as biomarker of human OTA exposure

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The aim of this paper was to identify the presence of OTA related DNA adduct in blood of Serbian man and women exposed to OTA via food. For this purpose we follow during four weeks Serbian families, analysing OTA and CIT content in food, DNA adduct in blood, and OTA metabolites in blood and urine. DNA adduct were analysed by postlabeling using the classical technique and using the new high sensitive method allowing us to identified by LC/ms/ms the C8-2'-deoxyguanosine OTA [1,2]. In parallel, we analysed DNA adduct in kidney and OTA metabolites in fluids biologic of male et female rat fed four weeks with increasing amount of OTA. In families having forebears suffering BEN, OTA contamination occurrence was higher compared to non BEN family [3,4]. Moreover food from BEN family contained CIT in contrast to food from non Ben families. C8dG OTA adduct was detected in rat kidney as well as in human blood. Ochratoxin B (OTB), open-ring ochratoxin A (OP-OA), 4-hydroxylated OTA, 10 hydroxylated OTA, OTalpha, OTbeta, two different OTHQ metabolites (quinone form), decarboxylated OTHQ (DC OTHQ) and GSH-OTA derivatives were identified in blood and urine of rat and Human. As for the DNA adducts, repartition of the metabolites are dependent of the sex and could be related to genetic polymorphism. Female rat and women excreted faster and more efficiently OTA and OTA metabolites, whereas male rat and man accumulated more OTA derivatives in blood. Altogether these data clearly shown that OTA is a complete carcinogen, and determination of OTA-DNA adduct is a relevant biomarker.

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Fusarium langsethiae infection in wheat, barley and oat under commercial cultivation

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A field survey was performed to study the infection and development of *Fusarium langsethiae* in wheat, barley and oat under commercial production. Sampling was completed in the 2008/2009 and 2009/2010 cropping seasons from fields within the counties of Shropshire and Staffordshire in the UK. Plants sampled (from tillering to harvest) were divided into roots, leaves, lower stem, upper stem and inflorescence/head sub-samples depending on the growth stage of the cereal. DNA was extracted and *F. langsethiae* DNA quantified using real-time PCR. Results showed oat to be the most susceptible cereal to *F. langsethiae* infection. *Fusarium langsethiae* DNA was not detected in the roots and seedlings of all three cereals suggesting *F. langsethiae* is not a root or seedling pathogen of cereals. Seemingly symptomless heads had high levels of *F. langsethiae* DNA, confirming speculation that *F. langsethiae* can be a symptomless pathogen of cereals.

Preliminary studies on the interaction between *Fusarium verticillioides* and *Fusarium graminearum* grown on different artificial media

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Fusarium verticillioides (Sacc.) Nirenb., belonging to the section Liseola, and *Fusarium graminearum* Schwabe (telemorph *Gibberella zeae* (Schwein.) Petch), being member of the section Discolor, both cause wide-spread diseases on maize in moderate as well as semi-tropical regions. These pathogens may commonly be isolated from maize kernels and their co-occurrence and produce of mycotoxins in maize plants has been well documented.
While *F. graminearum* causes red ear rot of maize most severely occurring in years and locations with frequent rainfalls and low temperatures during summer and early fall, *F. verticillioides* is frequently isolated from maize plants showing pink ear rot symptoms prevailing in drier and warmer climates. Both diseases are often accompanied by a high mycotoxin contamination in the kernels. 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. Only by understanding the nature of the interactions between the causal organisms of maize ear rot will it be possible to understand the consequences with respect to mycotoxin accumulation.
Therefore, we investigated growth behavior and mycotoxin production of different strains of both fungi cultivated on different artificial media under various experimental designs.
Having been cultivated on the same agar plate, both fungi showed similar growth rates on all media tested and no negative interaction was visible. Neither any inhibition zones nor growth retardation of either candidate could be detected even in the regions were the fungi overgrow each other. Mycotoxin production depended on the inoculation scheme characterized by placing agar discs from precultures of the corresponding fungi side by side either in a small or long distance. We observed an induction of trichothecene production of *F. graminearum* by *F. verticillioides* and a reduction of fumonisin production the other way round. These effects were strongly dependent on the medium used. Possible implications of these findings on disease development as well as food and feed safety will be discussed.

Diversity of *Penicillium expansum* isolates from apples related to *idh* sequence and patulin production

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Introduction: *Penicillium expansum*, the causal agent of blue mould rot of apples, is the main producer of the mycotoxin patulin in apples. Iso-epoxydon dehydrogenase, encoded by the idh gene, is a key enzyme in patulin biosynthesis. In this study, the diversity of *P. expansum* isolates was investigated through sequencing of the *idh* gene and analysis of the patulin production in order to relate sequence differences with patulin production capacity.
Materials and methods: On Malt Extract Agar, fungi were isolated from Jonagold apples originating in various orchards and from various moulded apples obtained from different sources in Belgium. Based on morphology, isolates of the genus *Penicillium* were selected and subjected to Random Amplification of Polymorphic DNA (RAPD) analysis. Nine reference strains were included as well. A similarity percentage of 90% was set to delimit separate clusters. Within each of these clusters, a representative isolate was selected for identification to species level by *beta-tubulin* gene sequencing. The presence of the *polygalacturonase* (*peg*) gene was verified by PCR (Marek et al., 2003). For a selection of isolates, patulin production capacity on Apple Puree Agar Medium (APAM) was analysed using ethyl acetate extraction followed by High Performance Liquid Chromatography (HPLC). Part of the *idh* sequence was determined, and sequences were aligned to detect possible polymorphisms.
Results and discussion: Morphological analysis of 106 fungal isolates resulted in selection of 55 *Penicillium* sp. RAPD analysis led to 35 separate clusters. By partial *beta-tubulin* gene sequencing of representative strains and deferring the identification results to the other members of the clusters, a total of 28 isolates and nine reference strains were identified as *P. expansum*. All *P. expansum* isolates were positive for *peg* PCR while all others were negative. This is in accordance with Marek et al. (2003). Preliminary results of partial idh sequencing and aligning indicate the presence of polymorphisms in the *idh* gene, e.g., for reference strain MUM 9919. Interestingly, this strain was reported by Baert (2007) as producing remarkably high amounts of patulin.
Conclusion: PCR using peg primers was confirmed to be specific for *P. expansum*. Alignment of the partial sequences of the *idh* gene showed that polymorphisms are present within *P. expansum*. Further study is necessary to relate *idh* gene sequence variations with patulin production capacity.

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Regulation of the alternariol biosynthesis in *Alternaria alternata*

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Alternaria moulds are ubiquitously present and commonly found on fruits, vegetables and cereals. One of the major toxins in *Alternaria alternata* is the mycotoxin alternariol. To date, only limited knowledge is available about the regulation of the biosynthesis of alternariol, especially under food relevant conditions. Depending on the foods fungi are exposed to osmotic stress due to a high concentration of different solutes or especially in fruits and vegetables they can encounter acid environments. The species *Alternaria alternata* is well adapted to these variable conditions. Strains isolated from vegetables were more sensitive to osmotic stress, but were well adapted to acid environments. In contrast strains from cereals behaved vice versa. In the current analysis it could be demonstrated that the alternariol biosynthesis is consistent during a wide pH range, whereas osmotic components like NaCl show a deep impact on the alternariol biosynthesis, resulting in complete inhibition already at low concentrations. High osmolarity in the environment is usually transmitted to the transcriptional level of downstream regulated genes by the HOG signal cascade, which is a MAP kinase transduction pathway. The inhibition of alternariol biosynthesis by changes in the osmolarity of the substrate might be regulated by this high osmolarity cascade. Expression of the MAP kinase genes hog1 and pbs2 and phosphorylation of the HOG1 protein were analyzed. A clear correlation between HOG1 phosphorylation and alternariol biosynthesis could be established. In addition, knock-out of hog1 by protoplast transformation lead to a non-toxin producing phenotype, which leads to the conclusion that regulation at MAPKKK level is essential for AOH biosynthesis. PacC is a key element in pH gene regulation. At alkaline pH-values, activation of PacC leads to suppression of acidity-expressed genes yet at acid pH-values, alkaline-expressed genes are suppressed. An increased mutual expression of the hog and pacC gene at pH 10 showed the connection between these two regulatory pathways. Analysis of alternariol biosynthesis, growth and gene expression of a pacC-knock-out transformant compared to the wild type and hog1 transformant were the scaffold to create a regulation scheme for alternariol biosynthesis. Currently a substractive suppressive hybridisation analysis is being performed to elucidate genes differentially expressed at osmotic stress conditions.

Diversity and gliotoxin production of *Aspergillus fumigatus* isolates from silage: A multidisciplinary approach

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The use of silage as animal farm forage during winter as well as (part of the) summer increases. Hence, the quality of silage has an important impact on animal health, welfare and production. Poor storage conditions can lead to undesirable growth of fungi and other microorganisms. Spoilage by fungi not only reduces the nutritional value of feed, but can also result in the production of mycotoxins and high amounts of allergenic spores. Especially the mycotoxins imply high risks for the animal health and due to possible carry-over into meat, milk and eggs also for the human health. The most prevalent moulds in spoiled maize silage are *Aspergillus*, *Penicillium*, *Monascus* and *Byssochlamys*. In this study, we focused on *Aspergillus fumigatus* isolates from silage. *A. fumigatus* is a saprotrophic and opportunistic pathogenic mould known to produce gliotoxin. As a result, the presence of gliotoxin in silage has been reported [1-3]. Although *A. fumigatus* is generally considered as a homogeneous species, strains may vary in both phenotypic and genotypic characteristics [4]. In order to examine this variability for *A. fumigatus* isolates from silage, phenotypic and genotypic techniques were combined in this study.

Although molecular identification based on the Internal Transcribed Spacer (ITS) region indicated that all isolates concerned *A. fumigatus*, phylogenetic analysis based on two different genes 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*. This subdivision was confirmed by means of Random Amplification of Polymorphic DNA (RAPD) analysis. Remarkably, UHPLC-MS/MS analysis showed a statistically significant ($P<0.05$) higher gliotoxin production for the *A. fumigatus* var. *ellipticus* isolates when grown on Yeast Extract Sucrose (YES) agar compared to the *A. fumigatus* var. *fumigatus* isolates from silage. However, PCR analysis indicated that this phenotypic difference in gliotoxin production between these two variants was not correlated with a genotypic lack or presence of the gliP gene, i.e., the gene coding for the key enzyme in gliotoxin production. This study emphasizes the need for multidisciplinary research and indicated *A. fumigatus* var. *ellipticus* isolates from silage as an important gliotoxin producer.

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FUM1, NOR-1 transcript genes and mycotoxins in animal feeds from South Africa associated with animal health disorders

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A link between fungi, mycotoxin levels in feeds and animal health and performance exists. Animal feed samples (n=100) will be analyzed for nor~1 (producing aflatoxins) and FUM1 (producing fumonisins) transcript genes, while aflatoxins (AF) and fumonisins (FB) will be quantified to establish a correlation between nor~1 and AF levels as well as FUM1 and fumonisins (FB). An *in silico* study will be done to clarify any transcriptional differences in different feeds. Furthermore, a feed sample will be spiked with or without an AF and FB-producers (singly or in combination) and biocontrol agent and stored for a month at different ecophysiological conditions (moisture and temperature). Sub-samples will be obtained weekly and analyzed to study nor~1 gene expression as influenced by environmental factors and to assess AF and FB production in feeds. Lastly, a toxicological study will be conducted in which toxin extracts from stored feeds will be tested on animal cell lines to determine cell viability/proliferation, cytokine production and barrier function. Microarray technology will be applied to assess effects at quarterly level, while flow cytometry will also be applied to determine cell death (apoptosis and necrosis). The study may assist in developing a more accurate risk assessment of AF and FB, their health effects, while management approaches can be optimized to minimize mycotoxin contamination of feeds.

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In vivo efficacy testing of mycotoxin binders in poultry by means of a toxicokinetic study according to EFSA guidelines

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Mycotoxins are of great concern in the poultry industry, because of their economical impact and the consumers health risk. The occurrence of mycotoxins in feedstuffs in Western-Europe is very high, up to 82% [1]. Therefore, a lot of methods for detoxifying mycotoxins have been developed. Mycotoxin binders are the most commonly used and seems to be the most promising way of counteracting the effects of mycotoxins. To evaluate the efficacy of these mycotoxin binders, the European Food Safety Authority (EFSA) stipulates that besides *in vitro* tests, also *in vivo* tests are mandatory. Moreover, these *in vivo* tests should not be based on non-specific parameters such as growth rate and feed conversion, but on ADME studies (absorption, distribution, metabolization and excretion) [2]. These ADME studies, or toxicokinetic studies, are mainly based on the mycotoxin plasma concentration-time profile after oral intake of the mycotoxin with or without binder.
In this study we evaluated the effect of two commercially available mycotoxin binders on different toxicokinetic parameters of T-2 toxin, deoxynivalenol (DON) and zearalenon (ZEA). Thirty-six broiler chickens, 17-days old, were divided into six different groups as follows:

Group 1: T-2/DON + BINDER 1	Group 4: ZEA + BINDER 1
Group 2: T-2/DON + BINDER 2	Group 5: ZEA + BINDER 2
Group 3: T-2/DON	Group 6: ZEA

After a one-week acclimatisation period, the broilers were administered an oral bolus of toxin, with or without binder. Next, blood was withdrawn from the *vena metatarsea plantaris* at different time points. Plasma was analyzed by a validated LC-MS/MS method for DON, T-2 and ZEA [3]. To evaluate the efficacy of two mycotoxin binders, different toxicokinetic parameters were calculated, e.g. area under the plasma concentration-time curve ($AUC_{0->\infty}$) (WinNonlin 5.0.1, Pharsight Corp., USA). Results will be presented at the conference.

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Influence of light on growth and mycotoxin biosynthesis of food relevant fungi

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Mycotoxins are toxic secondary metabolites of several food relevant filamentous fungi, like *Aspergillus*, *Penicillium*, *Fusarium* and *Alternaria*. The influence of light of varying wavelength on growth and mycotoxin biosynthesis of representatives of these species has been analysed. Generally the Penicillia seem to be more sensitive to light treatment than other fungal species. Interestingly wavelengths from both sides of the spectrum, e.g. red (long wavelength, 627 nm) and blue (short wavelength 470 – 455 nm) had the strongest inhibitory effects on growth and mycotoxin biosynthesis. Blue light generally had a stronger effect. Light of moderate wavelength, 590 to 530 nm (yellow to green) had more positive than negative influences on growth or toxin biosynthesis compared to the control (dark incubation). On one hand the inhibiting light effect was dependent on the growth medium. In contrast to malt extract medium (MEA), YES medium, as an especially rich nutrient medium, had an attenuating effect on the reactivity against light while the tendency of the response was the same in both media. On the other hand, the light intensity had a strong influence on how the fungus reacts. Depending on the resistance of the species a complete cessation of growth and/or an inhibition of mycotoxin biosynthesis could be achieved. Interestingly some kinds of secondary metabolites seem to have some protective effect against light irradiation and could be seen as a kind of adaptation mechanism. Examples are the mutual regulation of two mycotoxins ochratoxin A and citrinin which can be produced simultaneously by *P. verrucosum*. Citrinin was produced essentially under light conditions which inhibited ochratoxin A biosynthesis. Also a derivative of ochratoxin β, whose biosynthesis by *A. carbonarius* was strongly increased under blue light when the biosynthesis of ochratoxin A was ceased, may have some protective, light absorbing effects which afford the growth of this strain on sun exposed grapes. Light has a growth stalling effect on aerial mycelia but a deactivating effect on spores. If a non-growing colony under light is shifted to the dark it immediately grows normally, whereas 97% of spores which were incubated for 24 h under blue light were no longer able to germinate. Again the spores of the Aspergilli were much more resistant.

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Influence of mycotoxin detoxifying agents on the plasma pharmacokinetics and tissue residues of amoxicillin and oxytetracyclin in poultry

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Mycotoxin-detoxifying agents are frequently used feed additives for reduction of mycotoxicoses. Depending on their mode of action, the detoxifiers are divided in two different classes: adsorbing and biotransforming agents. The first ones are able to bind mycotoxins, the second ones have the ability to degrade mycotoxins. In poultry, amoxicillin and oxytetracyclin are frequently administrated in the drinking water for the treatment of bacterial diseases. Mycotoxin-detoxifying agents possibly could interact with the pharmacokinetics of these antibiotics.
To confirm this hypothesis, following experiment was conducted. Forty-eight 1-day-old Ross chicks were brooded under standard husbandry conditions. After one week acclimatization the animals were divided into six groups, each group consisting of eight animals. The chickens were fed diets containing no detoxifying agent (group 1 and 2), an adsorbing agent (group 3 and 4) or a biotransforming agent (group 5 and 6). After three weeks of feeding, a kinetic study of oxytetracyclin was performed in group 1, 3 and 5 (Table 1).

Table 1. Timetable of the critical events of the animal experiments with oxytetracyclin

Day in the study	D28	D29-33	D34	D35
8 a.m.	Oral bolus oxytetracyclin (100 mg/kg B.W.), followed by a kinetic study during 24 hours	Oral bolus oxytetracyclin (50 mg/kg B.W.) Oxytetracyclin in water (100 mg oxytetracyclin/kg B.W.)	Oral bolus oxytetracyclin (50 mg/kg B.W.) Refreshing of the medicated water (100 mg oxytetracyclin/kg B.W.)	Euthanasia of the animals 16 hours after withdrawal of medicated water and collection of liver and kidneys
8 p.m.		Refreshing of the medicated water (100 mg oxytetracyclin/kg B.W.)	Unmedicated water	

A similar experiment was performed for amoxicillin in group 2, 4 and 6 (Table 2).

Table 2. Timetable of the critical events of the animal experiments with amoxicillin

Day in the study	D28	D29-31	D32	D33
8 a.m.	Oral bolus amoxicillin (20 mg/kg B.W.), followed by a kinetic study during 10 hours	Oral bolus amoxicillin (10 mg/kg B.W.) Refreshing of the medicated water (20 mg amoxicillin/kg B.W.)	Oral bolus amoxicillin (10 mg/kg B.W.) Refreshing of the medicated water (20 mg amoxicillin/kg B.W.)	Euthanasia of the animals 12 hours after withdrawal of medicated water and collection of liver and kidneys
8 p.m.	20 mg amoxicillin/kg B.W. added in the water	Refreshing of the medicated water (20 mg amoxicillin/kg B.W.)	Unmedicated water	

The concentration of both antibiotics in plasma and tissues was determined by validated LC-MS/MS methods. Plasma pharmacokinetic parameters ($AUC_{0->\infty}$, $AUC_{0->t}$, C_{max}, T_{max},$T_{1/2}$) were calculated using WinNonlin. The results of this experiment will be presented at the conference.

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Acknowledgements

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Biological control of the phytopathogenic fungus *Fusarium culmorum* and its mycotoxin deoxynivalenol by earthworms

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A mesocosm based field study was conducted on plant pathogen repression as an ecosystem service provided by earthworms. The objective was to demonstrate that *Fusarium* biomass and deoxynivalenol (DON) content of wheat straw decrease substantially in the presence of earthworms in a practical cropping system.
Therefore, 48 mesocosms were established after harvest in the topsoil of a winter wheat field located near Hildesheim (Northern Germany), where conservation tillage is practiced since twenty years. Two different earthworm species of two different ecological groups were chosen: *Lumbricus terrestris* as a detritivore, anecic species and *Aporrectodea caliginosa* as a geophagous, endogeic species. The earthworms were exposed either separately (single-species) or combined (mixed-species) during an eight week period from mid August to mid October 2009 to artificially *Fusarium culmorum*-infected wheat straw highly contaminated with DON. A second set of mesocosms where earthworms fed on non-artificially infected wheat straw served as control. *Fusarium*-biomass was detected with a double antibody sandwich (DAS) ELISA, whereas DON content was determined by using a competitive ELISA. In contrast to *A. caliginosa*, *L. terrestris* incorporated *Fusarium*-infected and DON-contaminated wheat straw faster into the soil compared to control straw. Furthermore, the reduction of *Fusarium* biomass and DON-concentration in wheat straw was significantly higher in presence of *L. terrestris* than in treatments with *A. caliginosa* and without earthworms, where no significant differences could be measured between the *Fusarium* biomass and DON concentration in wheat straw. Thus, *A. caliginosa* seemed to be less important for the reduction of *Fusarium* biomass and DON concentration during the chosen time span.
We conclude that especially detritivorous earthworm species like *L. terrestris* are able to compensate possible drawbacks of conservation tillage by taking an active part in controlling phytopathogenic fungi and the degradation of their mycotoxins.

Abstracts of posters

Rapid aflatoxin results using NeoColumn™ aflatoxin direct read

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Introduction: The NeoColumn™ Aflatoxin Direct Read system combines an immunoaffinity column (IAC) for the detection of *Aspergillus* spp. toxins aflatoxin B1, B2, G1 and G2 with Neogen's custom-built DRF2100 fluorometer. Groundnuts, chilli and rice were successfully assayed using the system, offering users an effective means for compliance with aflatoxin EU import regulations. These results were equivalent or superior to the performance of a leading competitor IAC. Inter-laboratory studies were carried out using maize and peanut samples, demonstrating that the NeoColumn system is a robust and accurate tool for measuring aflatoxin in a sample. These findings support the data from a recent validation study under the requirements of the AOAC Research Institute Performance Tested Methods programme.

Materials and Methods: Spiked and naturally contaminated samples were extracted and assayed using AOAC 0.167 g and 0.5 g methods, prior to clean-up with NeoColumn or competitor IAC using a controlled flow rate. The IAC performance was evaluated using Neogen's DRF2100 fluorometer, calibrators and developer solution. Inter-laboratory studies at Neogen Europe and Neogen Corp. were carried out using the same certified reference materials.

Results: The data show that aflatoxin can be recovered from a variety of matrices, whether spiked or naturally contaminated. Use of the DRF2100 for fluorometry assay was effective in accurately determining aflatoxin recovery from multiple samples tested.

Discussion and Conclusion: Use of the NeoColumn Aflatoxin Direct Read IAC and DRF2100 fluorometer is a highly effective combination for the detection of aflatoxin in a variety of commercially important matrices. The Neogen system is rapid and portable, and offers a less expensive alternative to HPLC or ELISA for aflatoxin determination.

Keywords: NeoColumn™ Aflatoxin Direct Read, immunoaffinity column, fluorometry assay

Reference

Lupo, A.; Quain, A.; Fitzsimmonds, A.; Allan, A.B., 2011. Validation study of an immunoaffinity column coupled with solution fluorometry or HPLC for the detection of aflatoxin in peanuts and corn. J. AOAC Int. (accepted).

New selective SPE sorbent based on molecularly imprinted polymers for patulin

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Patulin [4-hydroxy-4H-furo[3,2-c]pyran-2(6H)-one] is a mycotoxin produced by a variety of molds, particularly *Aspergillus* and *Penicillium* species. It is commonly found in rotting apples, and the amount of patulin in apple products is generally viewed as a measure of the quality of the apples used in production.

Studies have shown that it is genotoxic. Several countries have instituted patulin restrictions in apple products. Member countries of the European Union have set maximum allowable levels of patulin at 50 µg/kg in fruit juices, 25 µg/kg in solid apple products, including apple compote, apple puree intended for direct consumption and 10 µg/kg in apple juice and solid apple products, including apple compote and apple puree, for infants and young children and in baby foods (European Commission Regulation (EC) 1881/2006 [1]).

Several analytical methods for the determination of Patulin have been developed in which a clean-up step is necessary and crucial. However, by the use of the classical methods of clean-up, the main matrix interferent, 5-Hydroxy-methylfurfural (HMF), is still present at a very high concentration, preventing a reliable quantitative Patulin determination. So there is an increasing need to improve both sensitivity and specificity of this key step of clean-up.

To propose an accurate solution, we have developed a new class of intelligent polymers based on molecularly imprinted polymers specific to Patulin. Molecularly Imprinted Polymer (MIP) is a synthetic material with artificially generated three-dimensional network able to specifically rebind a target molecule. MIP has the advantages to be not only highly selective and specific but also chemically and thermally stable, compatible with all solvents and cost-effective. This polymer is used as a powerful technique for clean-up and pre-concentration applications of Patulin. This study describes the solid phase extraction of Patulin from apple juice and apple puree utilizing a Molecularly Imprinted Polymer (MIP) SPE cartridge that is specific for Patulin.

Development of a LC-MS/MS method for the determination of zearalenone, deoxynivalenol and metabolites in blood serum

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Zearalenone (ZON) and deoxynivalenol (DON) are of special importance in animal nutrition due to the frequent contamination of cereal grains. Diagnosis of intoxications of farm animals might include the analysis of toxin residues in physiological samples. However, in the literature no analytical methods for the simultaneous determination of ZON, DON and their metabolites alpha-zearalenol, beta-zearalenol, zearalanone, alpha-zearalanol, beta-zearalanol and de-epoxy-DON in physiological samples can be found. In published methods for the determination of ZON or DON and metabolites HPLC coupled with UV-, fluorescence- or MS-detectors was used, whereas sample preparation included clean up with solid phase extraction (SPE) or immunoaffinity columns (IAC). The disadvantages of the HPLC methods compared to LC-MS/MS (liquid chromatography - tandem mass spectrometry) are the relatively low selectivity and sensitivity. Furthermore, they require cost and time intensive sample preparation. Therefore, the aim was to develop a selective and sensitive LC-MS/MS method including an economic sample preparation for the determination of ZON, DON and their metabolites in porcine blood serum.
For sample preparation four different materials were tested: Chem Elut™ cartridges (Varian) are packed with diatomaceous earth using the principle of solid-supported-liquid-liquid-extraction (SSLLE) and were used according to Valenta et al. (2003) with slight modifications. Strata™ X cartridges (Phenomenex) containing polymer-based C_{18} SPE sorbent and Strata™ C_{18}-U cartridges (Phenomenex) containing silica-based SPE sorbent were used according to manufacturer's prescription. The QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) method is based upon functionalized sorbents with primary and secondary amines (PSA) and was used according to a method of Plössel et al. (2006) with slight modifications. All methods included incubation with beta-glucuronidase (Type H-2, Sigma) before extraction. For LC-MS/MS measurements a 4000 Qtrap (Applied Biosystems) in negative ESI (electrospray ionisation) mode coupled with HPLC (1200 Series, Agilent) was used. The separation was performed on a Pursuit™ XRs Ultra 2.8 (Varian) column. Best results were obtained using gradient elution, whereby the mobile phases consisted of water (eluent A) and methanol/acetonitril (70/30 v/v) (eluent B).

Preliminary results showed slight differences of the suitability of the different extraction and clean up materials for the analysis of blood serum containing ZON, DON and their metabolites. The QuEChERS method and the Strata™ C_{18}-U cartridges resulted in the best recoveries (40-78% and 54-101%) for ZON and the metabolites. For DON and de-epoxy-DON none of these materials showed satisfactory results so far, probably due to matrix effects. Therefore, further experiments including the use of internal standards (IS) are in progress.

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New approaches for screening of mycotoxins in babyfood – possibilities and challenges

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Mycotoxins are known to harm the health of humans and animals. They are known either as carcinogenic or otherwise cytotoxic and impair the immune system. Therefore, different countries have set regulations on mycotoxins. In the EU mycotoxin limits are harmonized in the regulation for contaminants in foodstuffs EC 1881/2006 of 19 December 2006 and the amended regulation EC 1126/2007 of 28 September 2007. In July 2010 two new analytical methods for measuring Aflatoxin B1 and Zearalenone in infant food have been adopted as European benchmark (EN 15850, EN 15851). Both methods are based on a immunoaffinity column cleanup HPLC-FLD detection.

In this paper we want to present the possibility to analyze Aflatoxin B1 (AFB1) and Zearalenone (ZON) at a comparable detection level implemented into a LC/MS/MS screening method. For our measurements the AB SCIEX Triple Quad™ 5500 LC/MS/MS System was used. In one single LC/MS/MS run of 13 minutes 18 compounds were detected; 12 of them in the positive ionization mode and 6 of them in the negative ionization mode. The crude extracts of different infant foods were just diluted and injected without any additional sample preparation. Detection limits of AFB1 and ZON were found to be comparable to the required values by EN standards. Reproducibility in matrix was found to be lower than 10%.

Additionally we want to demonstrate the approach of screening for mycotoxins at babyfood concentration levels with a TripleTOF™ 5600 System. Due to its high resolution capabilities matrix effects can be further discriminated. Additionally quantitation and high resolution library confirmation can be achieved with just one single injection.

Determination of zearalenone in edible oils by enzyme immunoassay

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The *Fusarium* mycotoxin zearalenone (ZEA) is frequently found as a contaminant in all major cereal grains and other plant materials. Because ZEA is highly lipophilic, it is transferred into, or even enriched in the oil fraction during the production of edible oils. Maize germ oil seems to be of particular importance, but other edible oils may also be contaminated with ZEA. ZEA in refined maize oil was originally regulated at a maximum level of 200 µg/kg by European Union Commission Regulation 1881/2006. This value was later doubled (400 µg/kg) by Commission Regulation 1126/2007, "in order to avoid a disruption of the market" because of higher ZEA levels in maize in the 2005/2006 harvests. Since 2007, the Commission made no further amendments of the 400 µg/kg maximum level. ZEA in edible oil from other plant sources is not regulated at all. Analytical methodology for ZEA in edible oil is directed by the requirements of extraction and extract purification. So far, gel permeation chromatography followed by LC-MS/MS, and alkaline liquid-liquid partitioning or solid-phase hydrazine-assisted clean-up with LC-FLD determination have been described. These methods are all costly, and some may have sensitivity problems. Therefore the aim of this study was to establish a more simple and sensitive routine enzyme immunoassay for ZEA in edible oils.

A competitive direct enzyme immunoassay (EIA) with a mean detection limit of the ZEA standard curve of 97±30 pg/ml and a IC50 410±72 pg/ml was used for detection. The alkaline liquid-liquid partitioning was used for sample preparation as described by Majerus et al. (2009): oil samples (2 g) were extracted with methanol + 1% aqueous ammonium carbaminate solution, pH 9 (9+1, v+v). The methanol phase was evaporated to dryness, the residue dissolved with acetonitrile/water (25/75), diluted at least 1:5 with phosphate buffered saline and analyzed by EIA. The detection limit for ZEA in oil was 2 ng/g. Mean recoveries of ZEA from artificially contaminated oil samples were 112-207% at spiking levels of 2-50 ng/g, and 64-90% at 100-1000 ng/g. In a first survey employing this EIA method, a total of 31 oil samples was purchased in 2010 from retail shops in the Giessen area (Germany). Sampled products included maize germ oil (n=7), walnut oil (n=5), peanut oil (n=2), safflower oil (n=2), olive oil (n=2), sesame oil (n=2), and each one oil sample made from sunflower, rapeseed, soy bean, truffle, pumpkin seed, hempseed, grape seed, linseed, almond, wheat germs, and a non-specified "pure plant oil". All maize germ oils were strongly positive for ZEA (31-106 ng/g), although none exceeded the maximum level. The majority of the other oils was also positive (15/24), although toxin concentrations were typically lower, at levels of 2-47 ng/g. It is concluded that the EIA method described here is well suited as a rapid screening method to determine ZEA in edible oil.

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The MTT test used for the evaluation of cytotoxicity of fungi isolated from maize

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Maize, along with wheat and rice, is one of the most commonly grown plant species in the world. When the size of fields is considered, it holds the second rank, just behind wheat. High number of crops and the possibility of full mechanization of the whole production process made it also very popular in Poland [1]. However, the popularity goes hand in hand not only with the importance of the enlargement of the harvested area, but also with the economic significance of diseases and pest inhabiting maize. The activities of the pest decrease the number of crops, as due to various cuts, bacteria as well as fungal spores can easily get into the plant, thus leading to numerous diseases. Pathogens, which are able to produce secondary metabolites, called mycotoxins, are especially dangerous. These substances have toxic properties and pose hazard to both human and animal health [2].
The analyzed material included maize naturally contaminated with fungi as well as healthy plants, that were free from disease symptoms. The material was collected from the experimental fields - ZDD in Swadzim in September 2009. The moulds were isolated from the whole plant, as well as from the cob, stem and sprout separately and further analyzed. Subsequently, the isolated fungi were incubated for two weeks at the temperature of 250°C and then put under thermal stress of two-week incubation at 40°C. The control consisted of Czapek medium (CZ). Cytotoxicity of the samples was evaluated using the MTT cytotoxicity test, with swine cells (SK), which are sensitive to a wide range of mycotoxins.
The control used in the MTT test was Czapek medium (CZ), which did not show any toxicity. Low cytotoxicity (+) was detected for the following species: *Fusarium moniliforme* 554, *Fusarium oxysporum* 463, *Fusarium moniliforme* 553, *Aureobasidium pullulans* 473, *Fusarium equiseti* 456, *Acremonium strictum* 473, *Fusarium moniliforme* 458, and *Nigrospora oryzae* 555; while medium toxicity (++) was found for: *Fusarium poae* 464, *Fusarium moniliforme* 457, and *Ustilago maydis* 458. Moreover, cytotoxicity changes were observed before and after the thermal stress applied (tab. 1).

Table 1: The influence of thermal stress on the toxicity level change of the isolated moulds

Cytotoxicity	Examined mould species
No cytotoxicity	*Acremonim strictum* 458, *Cladosporium cladosporioides* 473, *Verticillium* 473, *Epicoccum nigrum* 456, *Acremonium strictum* 456, *Alternaria alternata* 456, *Fusarium culmorum* 461, *Torula* 461, *Aureobasidium pullulans* 456, *Mucor circinelloides* 472, *Acremonium strictum* 558, *Verticillium* 458, *Cladosporium cladosporioides* 555
Increased cytotoxicity after the thermal stress application	*Fusarium moniliforme* 554, *Fusarium moniliforme* 458, *Fusarium miniliforme* 458, *Ustilago maydis* 458
Decreased cytotoxicity after the thermal stress application	*Fusarium poae* 464, *Fusarium oxysporum* 463, *Fusarium moniliforme* 457, *Aureobasidium pullulans* 473, *Fusarium moniliforme* 553, *Fusarium equiseti* 456, *Acremonium strictum* 473, *Nigrospora oryzae* 555

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Production and characterization of antibodies against the *Alternaria* mycotoxin altenuene

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Altenuene (ALT) is a mycotoxin produced by different species within the genus *Alternaria*. It is a member of the benzopyrone type of mycotoxins, and possesses toxic properties in many biological test systems with animal and mammalian cells.

Polyclonal antibodies against ALT were obtained from rabbits after immunization with ALT-keyhole limpet hemocyanin conjugate prepared by formaldehyde condensation. Using these antibodies and an ALT-bovine serum albumin conjugate, a competitive indirect immunoassay (EIA) was established. The antisera obtained from three rabbits were highly sensitive for ALT. The antisera of one rabbit enabled the most sensitive detection of ALT, with an IC_{50} level of the standard curve of 2.2 ng/ml. The EIA was specific for ALT and did not cross-react with other mycotoxins of the *Alternaria* group (alternariol, alternariol monomethyl ether). This is the first description of antibodies against ALT and the first development of an EIA for this mycotoxin. Application studies of the EIA for the determination of ALT in foods, complementary to previous EIAs for alternariol and tenuazonic acid, are under way.

Estrogenic activity of red clover (*Trifolium pratense*) extract in comparison to zearalenone tested by the E-SCREEN

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High dosage of estrogenic substances in feed, like the mycotoxin zearalenone (ZEA), negatively influences the fertility of production animals. Red clover (*Trifolium pratense*), a widely spread fodder crop, is a phytoestrogens-rich plant. The aim of this study was to establish a test system to compare the estrogenic activity of phytoestrogens and the mycotoxin zearalenone (ZEA). Therefore, the E-SCREEN described by Soto et al. (1995) was adapted. MCF-7 cells, human estrogen-sensitive tumor cells, were cultivated with red clover extract under estrogen-free conditions. ZEA (320 ng/ml) was used as positive control, estrogen-free medium as negative control. Red clover extract together with ZEA was tested to study, if red clover extract affects the estrogenic activity of ZEA. Cell proliferation was measured photometrically using the WST-1 reagent. The data were analyzed by comparing the absorbance of the substance with that of the positive control. Cell proliferation of MCF-7 cells increased until a maximum value of 150% in the presence of red clover extract and was concentration dependent. Red clover extract had an additive effect on proliferation of ZEA treated cells (Figure 1).

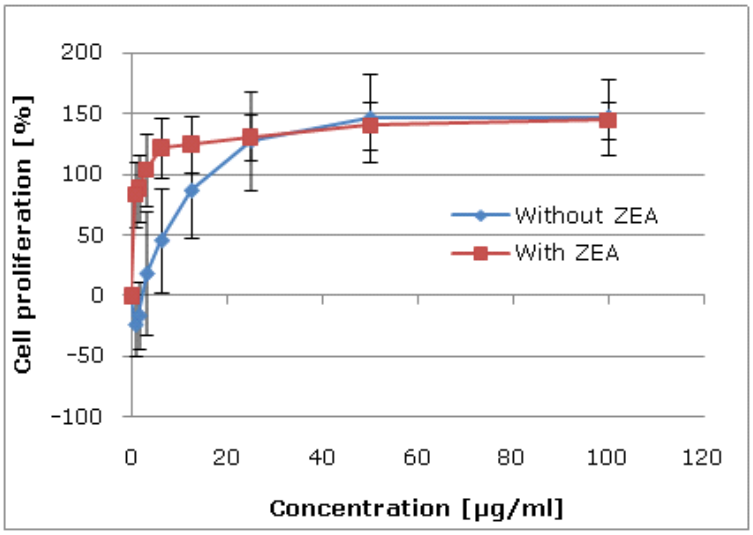

Figure 1: Proliferative effects of red clover extract on MCF-7 cells alone and in combination with ZEA. Data represent mean cell proliferation in % of positive control (ZEA) ± standard deviation from four independent experiments. 100% = cell proliferation in the presence of 320 ng/ml ZEA.

It was shown that the estrogenic activity of red clover extract in concentrations of 25-100 µg/ml was even higher than that of 320 ng/ml ZEA. The test system enables the rapid screening of phytoestrogens that are relevant in feedstuffs.

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Method validation study for the determination of trichothecenes T-2 and HT-2-toxin in cereals and cereal products by LC-MS/MS

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Analytical methods for the determination of HT-2- and T2-toxin in cereals and cereal products were typically based on gas chromatography with electron capture or mass spectrometric detection (MS) in former times. Due to the limited volatility of the toxins and the laborious sample preparation this methodology suffered some disadvantages. In the more recent past methods employing high performance liquid chromatography and mass spectrometric detection gained more importance and became introduced to be a common analytical tool in control laboratories.

Because there is a strong need in official food control for the confirmatory analysis of trichothecenes a rapid and economic method for the determination and monitoring of T-2- and HT-2-toxin in cereals and cereal flour was developed. The method is based on a solid phase filtration (SPF) clean-up followed by liquid chromatographic separation on a reversed phase-column and tandem mass spectrometric detection.

The method was tested in an interlaboratory study conducted with 14 laboratories according to the German Food and Feed Code, Section 64 (Lebensmittel- und Futtermittelgesetzbuch–LFGB, § 64). Five materials were distributed and analysed following the harmonised protocol. On contrary to other method validation studies the participants were allowed to use their own optimized LC-MS/MS conditions regarding the choice of ionization mode and mass transitions for quantification of the analytes. Some performance characteristics such as sensitivity for the toxins and the corresponding labelled internal standards were prescribed.

Data obtained in the collaborative trial and the contemporaneous proficiency testing are presented and discussed. Assessment of the precision data and the comparably results of the proficiency testing indicates the suitability of the validated method for official control purposes.

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Zearalenone in edible oil - a method comparison study

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Zearalenone (ZEN) is a mycotoxin produced by *Fusarium* spp. on a variety of cereals. Due to its lipophilicity considerable amounts of ZEN were found in edible oils, especially in maize oil. Toxicological studies revealed estrogenic and teratogenic effects on mammals, thus, EU-maximum levels were established for different foods ranging from 20 µg/kg (infant foods) up to 400 µg/kg (refined maize oil).

Currently, there is no standard procedure available for monitoring the maize oil maximum level. Existing methods for cereal-based products cannot be simply transferred to edible oils due to the incompatibility of organic solvents with immunoaffinity columns. Therefore, many efforts were made to overcome these problems leading to extraction/clean-up methods based on size exclusion chromatography [1], L/L-partitioning (with pH-value adjustment) [2], and recently, a solid-phase extraction of ZEN by a hydrazine-functionalised polymer resin [3].

In 2010 an interlaboratory comparison study (ILC) for the quantification of ZEN in three maize oil samples was organised by BAM. A total of 10 laboratories from Germany took part in the ILC applying extraction/clean-up methods of their own choice. All participants used HPLC analysis combined to fluorescence (FLD) or MS/MS detection. Whereas two maize oil samples possessed natural ZEN-contents of < 100 µg/kg, a third (blank) sample was spiked up to a total ZEN-content of 630 µg/kg.

The ILC results were statistically evaluated based on ISO 5725-2 (data evaluation after outlier rejection) and DIN 38402 A45 (robust statistics). As expected, the HPLC-MS/MS analysis was shown to be more selective than HPLC-FLD. The use of [$^{13}C_{18}$]-ZEN as isotopic-labelled internal standard makes HPLC-MS/MS the method of choice. However, HPLC-FLD is an appropriate alternative but requires an efficient clean-up. For that, the method according to [3] was seen to be very promising to remove fluorescence-active matrix compounds.

The results of the ILC can contribute to facilitate the method development and validation process. Considering the current legal and scientific situation with respect to ZEN in edible oils, reliable certified reference materials (CRM) are urgently needed. Therefore, one of the next CRM projects of BAM is focused on ZEN in refined maize oil.

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LC-MS-MS-determination of beauvericin and enniatins (A, A1, B and B1) and their occurrence in food

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Introduction: In 2010 the European Commission made a request for an EFSA opinion for a scientific opinion on the risks to human health related to the presence of beauvericin and enniatins in food [1]. To receive data for these mycotoxins as well as for other *Fusarium* toxins we implemented the five toxins into the LC-MS-MS-Multi-Mycotoxin-Method for unpolar mycotoxins [2].
Besides the occurrence of Beauvericin and the Enniatins A, A1, B and B1 in food the poster describes some analytical details, including some problems with the purity of the analytical standards and some validation data.

Results: All together we examined 380 different food samples. 220 (58%) of the samples contained Enniatin B up to 2610 µg/kg, 208 (55%) of the samples contained Enniatin B1 up to 1550 µg/kg. Enniatin A and Enniatin A1 were found in far less samples (14% respectively 12%) and in distinctly lower levels. Beauvericin was found very often and in comparatively high levels up to 216 µg/kg in dried figs.
The following table shows a summary of the most important results:

matrix	number of samples	Beauvericin		Enniatin A		Enniatin A1		Enniatin B		Enniatin B1	
		>RL	Max	>RL	Max	>RL	Max	>RL	Max	>RL	Max
rye and rye products	42		-	26%	19	71%	114	98%	1360	98%	519
wheat and wheat products	28	36%	18	36%	32	68%	151	100%	575	96%	494
durum wheat	5		-		-		-	100%	23	60%	9
corn and corn products	20	45%	126	5%	8	5%	5	20%	28	15%	18
spelt	8		-		-	13%	7	63%	52	63%	28
oat and oat products	53	17%	38	11%	8	32%	38	96%	146	87%	123
malting barley	1		-	100%	58	100%	392	100%	2610	100%	1550
barley malt	10	10%	8	90%	32	100%	156	100%	853	100%	585
wheat malt	1		-	100%	7	100%	33	100%	136	100%	107
flour for bread	5		-		-	80%	9	100%	214	100%	61
rye bread	56		-	4%	22	61%	114	98%	690	93%	400
sesame	28	14%	17		-		-		-		-
figs, dried	17	94%	216		-		-		-		-
raisins, sultanas	14		-	7%	7		-		-		-
pears, dried	12		-	8%	5	8%	8		-	8%	7

>RL: Percentage of samples with values above the reporting level of 4 µg/kg

Max: maximum value in µg/kg

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Synthesis of masked *Alternaria* toxins

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The mycotoxins alternariol (**AOH**) and alternariol-9-methyl ether (**AME**) are produced by fungi of the genus *Alternaria*. Glycoconjugates of these toxins (glucosides, glucuronides) are suggested to be formed during phase II detoxification, which was shown for AOH and AME glucuronides by Pfeiffer et al. [1] for hepatic and extrahepatic tissues. Detection and quantification of these metabolites is difficult or even not possible due to lack of reference materials.
Therefore we decided to develop reliable strategies for the synthesis of such compounds. Since AOH and AME contain more than one hydroxyl group, which are possible sites for glycosylation, different products would be obtained by just glycosylating the natural forms of these toxins. So it was reasonable to prepare AOH and AME derivatives, which are selectively protected to avoid complex product mixtures during glycosylation.
Starting from 1-bromo-3,5-dimethoxybenzene (**1**) and orcinol (**2**) two different derivatives were synthesized, alternariol-7,9-dibenzyl ether (**ADBE**) and alternariol-7,9-dimethyl ether (**ADME**). These compounds allow for selective conjugations at the free hydroxyl group or can be converted to AOH, AME and derivatives protected at position 3 (fig. 1).

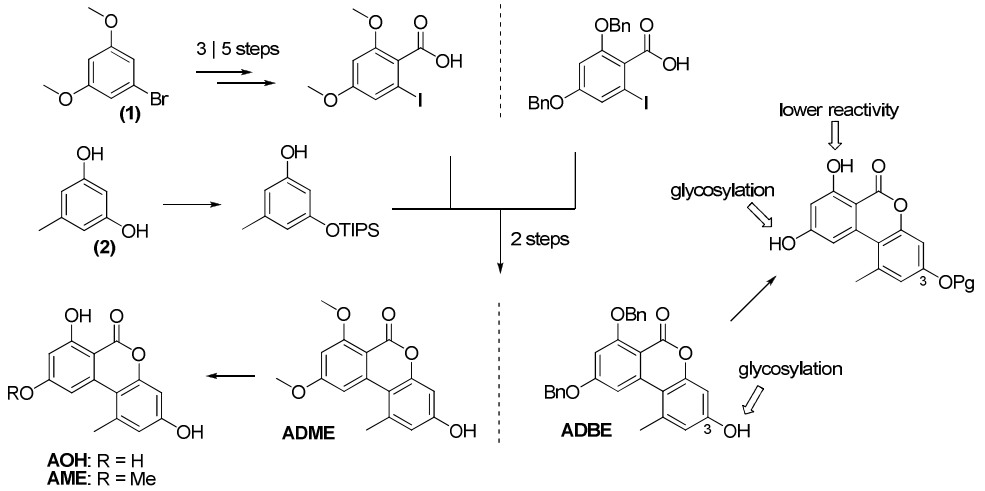

Figure 1: Synthesis of ADME, ADBE, AOH and AME
(Bn = benzyl, TIPS = triisopropylsilyl, Pg = protective group)

Glycosylation of these derivatives leads to all possible isomers of AOH and AME glucosides as well as glucuronides. The first glycoconjugates were synthesized using optimized procedures and final deprotections are currently under investigation.

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Simultaneous determination of mycotoxins aflatoxin M1 and ochratoxin A in milk and milk products

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The determination of Afaltoxin M1 and Ochratoxin A in one method is presented. Aflatoxin M1 is a metabolite of the Aflatoxin B1 and can be found in milk and milk products.
Ochratoxin A is investigated to get a survey, if there is any contamination of consumer milk and milk products. If cattel are exposed to a "high Ochratoxin A diet" the mycotoxin is not completely degraded by the known metabolism in the abomasum of ruminants.
An assortment of milk and milk products, i. e. fresh milk, UHT-milk, cheese, butter, and milk based infant formula, was investigated on Aflatoxin M1 and Orchatoxin A.
The determination is similar to ASU § 64 LFGB 15.03-1 or 15.00-1/2, i.e. a clean up with immunoaffinity columns (IAC) followed by an HPLC-FLD.
The various products have slight differences in sample preparation.
Fresh milk is sterilized prior to extraction to get better recovery rates of Ochratoxin A, UHT-milk is ready-to-use for extraction. Milk powder is prepared as described by Meucci et al..
Butter and cheese is first melted in a cabinet desiccator and then extracted with methanol/water 1:1.
After sample preparation and extraction the samples are transferred to a combinated Aflatoxin B1-G2 and Ochratoxin A IAC. Due to the cross reaction of Aflatoxin B1 and Aflatoxin M1, the latter is retarded.
Followed by a few preparation steps the eluate is ready for HPLC injection. The mycotoxins are detected by Fluorescence spectroscopy.
The LODs of Aflatoxin M1 and Ochratoxin A are 0,008 and 0,005 µg/kg, respectively. The LOQ are 0,024 and 0,014 µg/kg.
Recovery rates are between 75 – 105% for Aflatoxin M1 and 75 – 85% for Ochratoxin A.
A quantity of the given matrices was investigated. Aflatoxin M1 was in no sample detectable, Ochratoxin A was in a few samples in low amounts detected.

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Simultaneous determination of four *Fusarium* mycotoxins in baby food using DZT MS-PREP®

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R-Biopharm Rhône Ltd

Fusarium mycotoxins can be found in a variety of commodities when foods are stored under adverse conditions including temperature and humidity. Legislation for mycotoxins has recently increased to incorporate additional matrices and toxins, therefore resulting in an increased demand for faster and less labour intensive tests.
Immunoaffinity columns are rapidly becoming the routine standard method of choice for complying with regulatory mycotoxin analysis however there is a growing need for multi-mycotoxin analysis using a single extraction method. In response, R-Biopharm Rhone has produced a multi-toxin immunoaffinity column, DZT MS-PREP®, which enables the isolation and concentration of four commonly occurring *Fusarium* mycotoxins; deoxynivalenol, zearalenone, T-2 and HT-2. The advantages of this new immunoaffinity column are that only one sample preparation method and one single LC-MS/Ms run is required for quantifying all four mycotoxins therefore having greater sample throughput and a reduction in the use of solvents and consumables.
The method was validated in-house using DZT MS-PREP® to test various baby food samples. Recovery data ranged from 90 - 109% (RSD 0.3 - 3.1%) for column repeatability, while recovery data ranged from 84 - 106% (RSD 2.7 - 5.6%) for method repeatability. DZT MS-PREP® was shown to perform in line with current EU recommendations whilst providing a rapid and robust method of analysis enabling accurate, low level quantification of all 4 mycotoxins in baby food samples.

Synthesis of zearalenol mimics and masked mycotoxins

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The rapid global re-emergence of *Fusarium* head blight disease of wheat and barley in the last decade along with contamination of grains with mycotoxins attributable to the disease have spurred basic research on the fungal causal agent. As a result, *Fusarium graminearum* quickly has become one of the most intensively studied fungal plant pathogens. This work focuses on the *Fusarium* mycotoxin zearalenone, especially its metabolites α- and β-zearalenol and the synthesis of α,β-zearalenol-4-glycosides, which are formed during phase II detoxification, one of the plant's regulatory mechanisms (fig. 1).

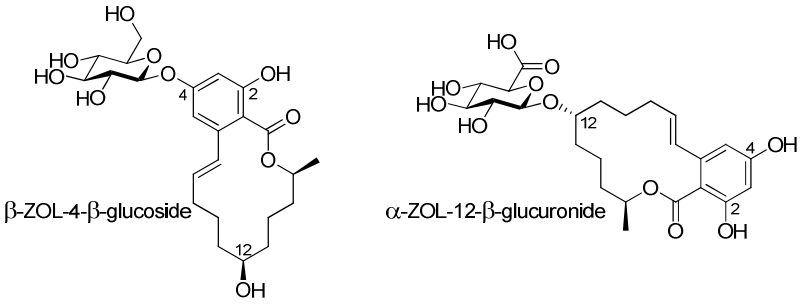

Figure 1: Examples of ZOL-glycosides

These conjugates are usually not detected using standard protocols and quantification is not possible due to lack of reference materials. Therefore we decided to mimic and optimize glycosylation procedures on model compounds. These mimics were synthesized and used for investigating two different strategies for regioselective synthesis of ZOL-glycosides (fig. 2).

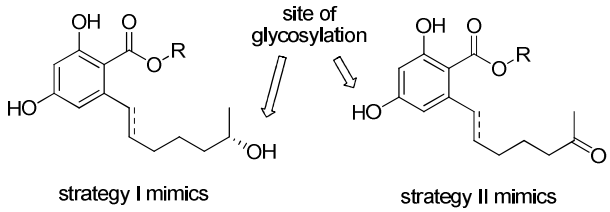

Figure 2: Mimics for the synthesis of ZOL-conjugates

Direct glycosylation of ZOL at the aliphatic OH-group due to a higher nucleophilicity compared to the OH-group in position 4 (see fig. 1) is planned within strategy I. Alternatively phenolic ZOL-glycosides can be synthesized by reaction of ZON with glucosyl or glucuronyl donors followed by ketone reduction using sodium borohydride and final deprotection (strategy II).
Applying these strategies on the prepared mycotoxin mimics different optimized procedures were developed and used for the synthesis of ZOL-glucosides and ZOL-glucuronides. Synthesis of ZOL-sulfates using these strategies is currently under investigation.

Fluctuation of ELISA for *Fusarium* toxins detection in maize

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Mycotoxins are secondary metabolites produced by fungi, which have toxic effects for humans and animals. In Europe, *Fusarium verticillioides* and *Fasurium graminearum* are the major fungi which contaminated in maize. They produce several mycotoxins such as trichothecene, fumonisin, and zearalenone. ELISA test kits are rapid, easy-to-use, and become the most widely used method. However, cross-reactivity is a disadvantage of ELISA and results in underestimation or overestimation of the level detected. Comparison of instrument method and ELISA has been reported for samples both naturally and artificially contaminated with mycotoxins (Sydenham et al., 1996; Bolduan et al., 2009). They found good correlation between these methods using linear regression analysis. However, Bland and Altman (1999) suggested that a high correlation is no guarantee of good agreement between methods. They suggested a plot of the difference against the average of the two methods of measurement.

Therefore, in this study, maize plants were inoculated with *F. verticillioides* and *F. graminearum* in experimental fields in Germany from 2006 to 2009. Then, mycotoxins including, deoxynivalenol (157 samples), fumonisin (126 samples), and zearalenone (145 samples) were measured by ELISA at three laboratories and by LC-MS/MS at one laboratory. The objective of this study was to evaluate the performance of the ELISA method compared with LC-MS/MS, used as reference method. Moreover, to investigate the fluctuation of ELISA, inter-laboratory results were also compared.

The results found that ELISA method produced either overestimation or underestimation when compared with LC-MS/MS. Moreover, the skill of the technician in applying on ELISA should be focused. Although, ELISA has an advantage on its having low cost and easily used

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$^{13}C_2$-Moniliformin: Synthesis of an isotopically labeled standard

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Moniliformin (MON) is a frequently occurring toxic secondary metabolite in cereals and maize worldwide which is produced by a number of *Fusarium* species, including the common corn pathogens *F. avenaceum*, *F. proliferatum* and *F. verticillioides* [1]. Naturally it occurs as sodium or potassium salt of 1-hydroxycyclobut-1-ene-3,4-dione and was first isolated and characterized by Cole et al. in 1973 [2] Toxicity studies with different animal species indicate that MON is a potent cardiotoxin. The molecular mechanism for the toxic effect of MON involves selective inhibition of mitochondrial pyruvate and alpha-ketoglutarate oxidations [3].
Due to the potential health risk for humans and animals caused by MON, the establishment of fast and reliable methods for the detection of this mycotoxin in different matrices is of importance. For this purpose the use of HPLC-MS is prevalent and well established. However, the use of HPLC-MS is mostly limited by the effect of matrix compounds on the ionisation of the analyte. To overcome this limitation the stable isotope dilution analysis by using isotopically labeled standards is the method of choice. However, until now there is no isotopically labeled MON commercially available.
Several synthetic routes to MON have been reported. The most recent synthesis was reported by Fétizon et al. in 1990 based on a [2+2]-cycloaddition of 2,3-dihydro-1,4-dioxin and dichloroketene via formation of a four-membered ring precursor. Hydrolysis of that cyclobutanone precursor yields MON [4].
Based on this straightforward approach we developed a modified strategy for the first synthesis of isotopically labeled MON by using trichloroacetylchloride-1,2-$^{13}C_2$ as ^{13}C source for isotope introduction (Figure 1).

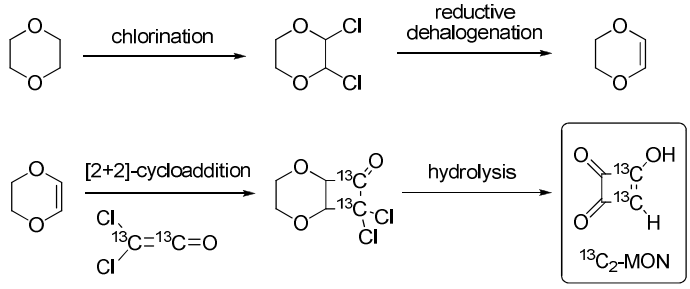

Figure 1: Strategy for the synthesis of $^{13}C_2$-Moniliformin.

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Determination of deoxynivalenol and T2 toxin degraded by *Lactobacillus acidophilus* K1 strain by use of ELISA method

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Using many available methods for physical and chemical degradation of food products contaminated with mycotoxins is limited because of safety, the probable loss of nutritional value and the limited effectiveness and costs of the application. Many studies confirm that bacteria have the ability to degrade different mycotoxins, so they can become an alternative to other methods of degradation of mycotoxins [Fuchs et al 2008].
However, in previous studies, mycotoxins HT-2, T-2, NIV and 3AcDON were poorly degraded by lactic acid bacteria. Reduction of HT-2 ranged from 0% to 10%, T-2 from 0% to 27%, NIV from 0% to 22%, while there was no degradation of 3AcDON [El-Nezami et al 2002]. Therefore, there is a need for further exploration of strains, effectively degrade these mycotoxins.
This study compared two analytical methods for detecting deoxynivalenol (DON) and T2 toxin produced by *Fusarium* species and evaluation degree of the biodegradation DON and T2 toxin by the live and dead cells of lactic fermentation bacteria *Lactobacillus acidophilus* K1 strain using ELISA method.
Degradation DON and T2-toxin was performed in 24 hour long incubation in phosphate buffered saline (PBS) as the base and various pH (from 4 to 7). Standards DON and T2 were dissolved in acetonitrile and added to PBS. The concentration of mycotoxins was marked with use of the direct competence ELISA tests - AgraQuant Test Kit (Romer Labs). Dead cells of bacteria were obtained by thermal inactivation in PBS. Gas chromatography was used to compare the results with ELISA method. The clearing of samples for GCMS was made according the SPE method (Valle-Algarra et al. 2005).
The highest degradation of DON (41.2%) had live bacterial cells of *Lactobacillus acidophilus* K1 at pH 6.0, and degradation of T2-toxin (78.8%) at pH 7.0. Heat-inactivated bacterial cells generally degraded mycotoxins to a smaller degree than the live ones. However, at pH 5.0 reduced by 30.3% more T2-toxin and at pH 7.0 by 32.1% more DON than alive cells.
The biggest difference between the results of degradation mycotoxin T2 by *Lactobacillus acidophilus* K1 obtained by two analytical methods was 25.8%, and 17% for degradation of DON. The average difference between the results obtained by GC and by ELISA methods amounted to 13.4% in case of T2-toxin degradation and 10.6% for DON.

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Feasibility study on the development of a matrix reference material for patulin in apple by-products

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An essential requirement for a certified reference material is the stability of the analyte but also the stability of the matrix. Due to the toxicity of patulin, various studies reported so far have focused on the detoxification rather than the stabilisation of patulin. From these studies complementary information can be obtained to develop processes that can stabilise the apple matrix and minimize the degradation of patulin. Furthermore, limited information on the long term stability of patulin and apple products is available since apple products are generally consumed within 1 year after the harvest. From the microbiological point of view apple by-products are not considered as sensitive food (high sugar content and the low pH). Therefore, severe treatments are avoided because they would inevitably change the organoleptic characteristics. For this reason, all industrial processes used to prolong the shelf life are mild treatments, such as pasteurisation, concentration or freezing. These treatments do not significantly reduce patulin levels and fail to fully remove heat resistant fungi that could produce patulin in the finished apple by-products [1].
Since no data on studies on the stability of apple by-products and patulin after the application of more efficient stabilisation techniques such as autoclavation or freeze-drying have been published, a feasibility study for the production of apple by-products containing patulin was performed. Commercially available apple juice was freeze dried, but the product had a rubber-like consistency and was considered as unsuitable for further processing. Fresh apples were also minced and freeze-dried. The residual water content in the lyophilised material was deemed to be too high and, based on past experience, instability of the matrix was considered to be likely. Only commercially available apple puree could be successfully freeze-dried. The same material was also autoclaved and further investigated. The patulin analysis was performed (SPE purification and HPLC-UV detection) on these two candidate reference materials. Results indicate that the modified method developed in-house was inadequate, as changes in the matrix did not allow the quantification of patulin. The freeze-dried and the autoclaved material contained interferences as a compound is co-eluting with the patulin, in particular for the autoclaved material. The complexity of the chromatographic profiles hampered any further investigation on the stability of patulin. Finally, the stability of patulin in frozen apple puree was studied applying isochronous storage schemes. The material was proven to be stable so far over 3 months at -30°C and did not contain compounds interfering with the determination of patulin.

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Development, validation and field application of two optimized flow through multi-mycotoxin immunoassay formats to screen a variety of food matrices

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Immunoassays for mycotoxin detection has been regarded as valuable tool to complement the highly précised confirmatory analytical techniques such as liquid chromatography tandem mass spectrometry (LC-MS/MS). Global trade has obliged most countries to depend on one another for food and as a result exposed them to mycotoxin contaminated imported food products. If no proper control or monitoring is done at the port of entry, these contaminated food products would end-up in the food chain and would serve as possible hazard to human health. In developing countries in particular, where high levels of mycotoxins (fumonisins and aflatoxins) are frequently reported there is has been the need for fast reliable on-site screening assays so as to arrive at a quick decision on the acceptance or rejection of foodstuffs (both imported and locally produced).
Two immunochemical flow through assay formats (gel and membrane-based) were developed and validated for simultaneous detection of zearalenone, aflatoxin B1, deoxynivalenol, fumonisin B1 and ochratoxin A, in cassava flour, maize, peanut cake and peanut samples. Both methods combine a sample extraction, clean-up, dilution of the sample extract, pre-concentration and multi-immunodetection through enzymatic reaction of horse radish peroxidase (HRP). The visual detection limit for the membrane based assay, which is the lowest mycotoxin concentration resulting in no colour development, was 5 µg/kg, 700 µg/kg, 3 µg/kg and 175 µg/kg for AFB1, DON, OTA and ZON respectively. The gel based column assay format has cut-off limits at 3 µg/kg, 1 mg/kg, 200 µg/kg and 1 mg/kg for OTA, FB1, ZEN and DON respectively. The percentage false negative rate was less than 5% when naturally contaminated samples were analyzed using both formats. Both assay formats showed excellent applicability under tropical field conditions.

MIP - Molecular Imprinting Polymer: Neue Phasen für die Mycotoxin-Analytik

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Seit Jahren sind Festphasenextraktionstechniken über Silica - und Polymerphasen sowie mittels Immunoaffinität bekannt. MIP - Molecular Imprinting Polymer - Phasen besitzen wie die Immunoaffinitätssäulen eine hohe spezifische Bindung zu verschiedenen Analyten, haben aber eine wesentlich höhere Bestimmungsgrenze und sind in der Handhabung vergleichbar mit Festphasenextraktionskartuschen. Jetzt sind auch MIP – Phasen für die Mycotoxin Analytik verfügbar, welche eine hohe Selektivität wie Immunoaffinitätssäulen aufweisen aber auch die Robustheit von „normalen“ Silica - / Polymerphasen in einem Material vereint.

Multiplexed mycotoxin direct immunomagnetic assays using xMAP technology with phycoerythrin-mycotoxin conjugates

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The xMAP technology developed by Luminex is based on microsphere arrays. These polystyrene microspheres, more often referred to as beads, are internally dyed with fluorochromes. The paramagnetic beads (6.5 µm) used in the presented research are internally dyed with two or three fluorochromes to create up to 500 unique bead sets. The beads are excited with red light for classification. This creates the possibility for using just one reporter molecule, most often the fluorescent protein R-Phycoerythrin (R-PE), which is excited by green light.
The FlexMap 3D flow cytometer is the newest development in multiplexing capacity using xMAP technology. This instrument is a bead-dedicated flow cytometer capable of characterising 500 unique parameters in a single well, by unique combinations of two or three dyes. The read time for a 96-well plate measuring up to 100 parameters in a single well in 18 minutes.
The MagPix system is a new multiplexing planar array platform which is also based on the xMAP technology. The machine uses a monolayer of paramagnetic beads, captured by a magnetic source in a flow chamber, and LED lights combined with CCD imaging technology and thus technically deviates from the flow cytometry formats utilised in previous Luminex machines. It can detect 50 parameters in a single well. The measurement of a 96-well plate takes approximately one hour. Both suspension and planar array formats were used during the development of a fast and sensitive direct competitive inhibition immunoassay for the simultaneous detection of the mycotoxins Aflatoxin B1, Fumonisin B1, Deoxynivalenol, Ochratoxin A, Zearalenone and T-2 toxin in food and feed. For this direct immunoassay, the beads were coated with monoclonal antibodies and mycotoxins were conjugated to R-PE to create unique reporter molecules. This assay fits in the growing demand for multiplex testing and is more favourable in terms of labour, cost and hands-on time in comparison to single ELISA's and LC-MS. In the future this assay can be easily extended with other mycotoxins of interest, provided that relevant antibodies are available.

Reference

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Advanced synthesis of malonylglucosylated mycotoxins

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The so called masked mycotoxins are formed during phase II detoxification and detection as well as quantification is still a problem or even not possible due to lack of reference materials. Therefore these compounds get more and more in the focus of synthetic chemists.
It was suggested that malonylglucosylated mycotoxins (e.g. zearalenon-4-malonylglucoside, fig. 1) derive from the corresponding glucosides [1] via malonylation of the hydroxyl group at position 6 of the sugar molecule.

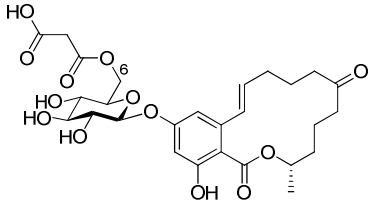

Figure 1: Structure of zearalenone-4-malonylglucoside (ZON4MalGlc)

For the synthesis of malonylglucosides special glycosyl donors have to be designed, because it is not possible to apply classic protective groups like acetyl or benzoyl. Deprotection of these esters under basic conditions would also cleave the malonyl group. So we decided to use a synthetic strategy, which was also applied for the synthesis of glucosides and glucuronides in our lab. By combination of benzyl ethers, one benzyl ester (at the malonyl moiety) and benzyloxycarbonyl protection of the hydroxyl group at position 2 of the glucose, diastereoselective glycosylation (β-selective) as well as deprotection under mild reaction conditions (palladium catalyzed hydrogenation) is possible (fig. 2).

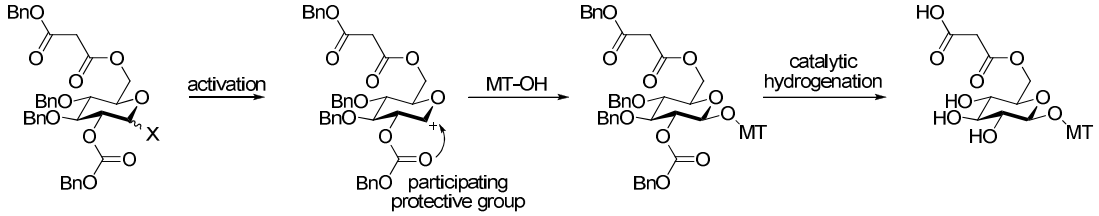

Figure 2: Synthesis of malonylglucosylated mycotoxins using new glycosyl donors
(Bn = benzyl, X = leaving group, MT-OH = mycotoxin)

Different analogs of these glycosyl donors were also prepared using other protective group strategies, and therefore deprotection is possible under variable reaction conditions. These methods were optimized and different leaving groups were applied for the synthesis of malonylglucosides.

Reference

[1] Berthiller, F.; Werner, U.; Sulyok, M.; Krska, R.; Hauser, M.-T.; Schuhmacher, R., 2006. Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) determination of phase II metabolites of the mycotoxin zearalenone in the model plant *Arabidopsis thaliana*. Food Additives and Contaminants, 23, 1194-1200.

Screening of barley and malt for *Fusarium* occurrence and mycotoxin contamination

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Field samples of barley from Bavaria and the resulting malts were tested for malting quality. Moreover, the grains and malts were assessed mycologically for fungal contamination and screened for toxins by LC-MS/MS [1].
The investidated samples showed visual symptoms at different frequencies and the mycological investigations detected a unique spectrum of fungal contamination.
In most of the barley samples, the type A trichothecenes T2- and HT2-toxin were detectable, whereas the type B trichothecenes deoxynivalenol, its acetyl derivatives and nivalenol occurred less frequently. In some samples, also the depsipeptides Enniatin A and Beauvericin could be detected.
After malting, the content of the *Fusarium* toxins often decreased, whereas some significant exceptions were observed.
Generally the contamination of barley with moulds is one of the most critical quality criteria. Fungal metabolites can compromise processing and consumption. Still visual symptoms, fungal contamination, detected mycotoxins and malting quality data did not relate easily. The dynamics in the complex context of field contamination will be further investigated to be better understood and controlled.

Reference

[1] Asam, S.; Rychlik, M., 2008. Studies on accuracy of trichothecene multitoxin analysis using stable isotope dilution assays, Mykotoxin Res., 23, 191-198.

Two new stable isotope dilution assays for ochratoxin A in human blood

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The mycotoxin ochratoxin A (7-carboxy-5-chloro-8-hydroxy-3,4-dihydro-3R-methyliso-coumarin-L-β-phenylalanine, OTA) is a metabolite produced by *Penicillium* (e.g. *veridicatum*) and *Aspergillus* (e.g. *ochraceus*) species on a wide variety of inaccurately stored foods. Consequently, OTA has been detected in cereals, coffee, cocoa, wine, beer, spices, and milk. Due to its nephrotoxic, carcinogenic, teratogenic and immunosuppressive properties, maximum residue limits have been established in the European Union e.g. for cereals, cereal products, dried vine fruits, coffee, wine, and spices.
For a risk evaluation of consumers' intake, two new stable isotope dilution assays were developed for the quantification of ochratoxin A in human blood samples. As internal standard, the recently synthesized [$^2H_5$]-OTA was applied [1]. The concentration of the unlabeled reference standard was determined by quantitative NMR. The methods applied two different sample extraction and clean-up procedures, i.e. liquid-liquid extraction with following immuno affinity chromatography (IA) as well as a dispersive solid phase extraction (DSPE) method. Detection was performed by LC-MS/MS. Additionally, a comparison of different blood drawing procedures revealed no differences for heparin plasma and serum whereas citrate plasma gave significant lower results for the mycotoxin. Limits of detection (LOD: 0.02 ng/g (IA) vs. 0.03 ng/g (DSPE)), limits of quantification (LOQ: 0.07 ng/g (IA) vs. 0.08 ng/g (DSPE)), relative recovery (≥ 93%), precision and linearity indicated excellent performance of the developed methods.

Reference

[1] Lindenmeier, M.; Schieberle, P.; Rychlik, M., 2004. Quantitation of Ochratoxin A in Foods by a Stable Isotope Dilution Assay Using High-Performance Liquid Chromatography-Tandem Mass Spectrometry, J. Chromatogr. A, 1023, 57-66.

The advantage of fully stable ^{13}C-labeled internal standards in LC-MS/MS mycotoxin analysis

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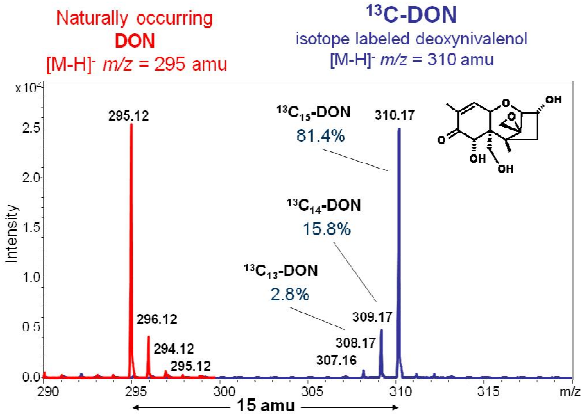
The LC-MS/MS technology popularity is constantly increasing. More and more laboratories are now using LC-MS/MS also for mycotoxin routine analysis. Nevertheless, a problem with LC-MS/MS can be interferences from matrix components leading to differences in analyte ionization. Ionization efficiencies can vary between matrix samples and pure standard calibrants. Hence, the mass spectrum shows different signal intensities and because of this the sample analyte peak cannot be compared to the calibration curve (made from pure standard calibrants) for concentration calculation. To overcome this ionization effect, internal standards have to be used. One possibility are isotope labeled standards, where one or a number of constituting atoms are replaced by a different stable isotope. A possible isotope can be ^{13}C. ^{13}C-labeled mycotoxins still have the same characteristics as their ^{12}C analogues, eluting at the same retention time in chromatography. When the detection is performed with mass spectrometry, both eluted forms are found and can be separated because of the mass difference between ^{12}C and ^{13}C mycotoxins. The ^{13}C peak, representing the known amount of ^{13}C labelled mycotoxin added can be used to calculate the unknown amount of the ^{12}C mycotoxin.



Full scan MS spectra of ($^{13}C_{15}$)-DON (Biopure) showing the isotopic distribution (blue line). Overlayed is the isotopic pattern of natural occuring DON (red line).

Using fully stable ^{13}C-labeled mycotoxins as internal standards has several advantages over deuterated (^{2}H) internal standards. Replacing ^{12}C by ^{13}C changes the total mass of the atom only slightly, while using Deuterium, the mass doubles, thus, ^{2}H labeled mycotoxins might show retention time shifts, resulting in less accurate LC-MS/MS results. This presentation will demonstrate how to use an internal standard. Furthermore, it will illustrate the importance of applying internal standards when running quantitative mycotoxin analyses on an LC-MS/MS system.

Production of partially hydrolyzed fumonisins B_1 as standards for determination of fumonisin biomarkers in biological samples

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Introduction: The mycotoxin fumonisin B1 (FB_1) can be cleaved to partially hydrolyzed FB_1 ($pHFB_1$) and hydrolyzed FB_1 (HFB_1) by removal of one or both tricarballylic acid side chains in the GI tract of animals (Shephard et al. 1994). The aim of this work was to produce standards of $pHFB_{1a}$ and $pHFB_{1b}$ (differing by the position of the remaining tricarballylic acid side chain) by partial enzymatic cleavage of FB_1 using FumD originating from *Sphingopyxis* sp. MTA144 (Heinl et al. 2010) and preparative HPLC.

Experimental: 10 mg of pure FB_1 were incubated with 100 µl of a fermentation supernatant, diluted 100-fold in FE buffer, of recombinant *Pichia pastoris* producing FumD (Heinl et al. 2010) at 37°C for 85 min. The enzyme was inactivated by boiling in a water bath for 10 min. Subsequently, the solution containing HFB_1, $pHFB_{1a}$, $pHFB_{1b}$ and residual FB_1 was diluted, filtered and used for preparative HPLC. Preparative HPLC was carried out on a Phenomenex Gemini-NX column (150 x 21.1 mm, 5 µm) in gradient elution mode using mobile phases composed of methanol, water and 0.1% acetic acid. 500 µl of fumonisin solution were injected and the compounds were separated at 16 ml/min within 11.5 min. Fractions containing HFB_1; $pHFB_{1a}$; $pHFB_{1a}$ + $pHFB_{1b}$; $pHFB_{1b}$ and FB_1 were collected and the mixed fraction containing both forms of pHFB1 was evaporated and re-injected. Finally, the combined fractions were evaporated almost to dryness on a rotary evaporator and the residual water was removed by freeze drying. The structures of $pHFB_{1a}$ and $pHFB_{1b}$ were elucidated by NMR analysis.

Results and discussion: NMR analysis confirmed the results obtained by Shephard et al. (1994), identifying $pHFB_{1b}$ as the isomer with the tricarballylic acid on C14. The partially hydrolyzed fumonisins could well be separated from FB_1 and HFB_1. However, baseline separation of $pHFB_{1a}$ and $pHFB_{1b}$ would require a flatter gradient resulting in longer run times. In order to keep the run time short, an intermediate fraction containing both $pHFB_{1a}$ and $pHFB_{1b}$ was collected and re-injected after evaporation. The yields of HFB_1, $pHFB_{1a}$, $pHFB_{1b}$ and FB_1 were 0.7, 3.0, 4.1 and 1.5 mg, respectively. Due to interconversion of $pHFB_{1a}$ and $pHFB_{1b}$ in solution, standards were stored at -20°C.

Conclusion: Standards of $pHFB_{1a}$ and $pHFB_{1b}$ are now available and can be used to determine the extent of hydrolysis of FB_1 and the concentrations of its partially hydrolyzed forms in biological samples.

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Purification and characterization of radicicol from fungal strains and development of a LC-MS/MS method for quantification of radicicol and its conjugates

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The antifungal antibiotic radicicol (RAD), also known as monorden, was first isolated from *Monosporium bonorden* [1]. RAD is also produced by other species such as *Humincola fuscoatra*, *Monocillium nordinii*, *Colletotrichum graminicola* or *Nectria radicicola* [2]. Regarding the mode of action it has been shown that RAD binds to and thereby inhibits the heat shock protein 90 (Hsp90), which shows ATPase activity in eukaryotic cells [3].
The first task of this work was the screening of different fungal strains for their utility to produce RAD. Various media and growth conditions were evaluated. In this study we used mainly isolates of *Nectria radicicola*, which is a major root rot pathogen of ginseng. Different extraction methods were examined for a quantitative recovery of RAD. Raw extracts were purified using normal phase flash chromatography and concurrent reversed phase preparative chromatography. The substance was obtained in the gram-range with purities >97%. RAD is light sensitive, which has been tested in liquid media. The results show decreasing concentration of RAD the longer it is exposed to light. Furthermore, purified RAD was used in growth assays to test inhibitory levels on plants and yeasts. The metabolization of RAD by plants was evaluated with a LC-MS/MS method, screening for 25 RAD-conjugates, predicted to be potential metabolites of RAD. For the first time, a glucoside of RAD was found in RAD treated *Arabidopsis*. A glucosyltransferase, known to glucosylate the structurally similar mycotoxin zearalenone, was expressed in yeast and used to produce RAD-glucoside. HR-MS and NMR measurements of the purified compound confirmed its sum formula and structure. Finally, we used the LC-MS/MS method to screen for RAD contamination in different food and feed samples. In 2 of 26 ginseng root food supplements, RAD was found in concentrations up to 300 ng/g. Maize silages were also found to contain RAD up to 98 ng/g. In maize samples collected from various parts of Africa, RAD was found up to 37 ng/g. No conjugates of RAD were found to occur in naturally contaminated samples.

References

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[2] White, N.; Chilvers, G.; Evans, G., 1962. Antifungal activity of *Cylindrocarpon radicicola* Wr. Nature, 195, 406-407.

[3] Wicklow, D.T.; Jordan, A.M.; Gloer, J.B., 2009. Antifungal metabolites (monorden, monocillins I, II, III) from *Colletotrichum graminicola*, a systemicvascular pathogen of maize. Mycol Res, 113 (12), 1433–1442.

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Comparison of Immunoaffinity Columns (IAC) of classic and miniature/spin format analysis of aflatoxin and DON

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We are highly interested in the influence of column geometry on the performance of immunoaffinity columns (IAC) which are widely utilized in sample preparation. Specifically, the effect of two different column diameters on both the recovery rate and reproducibility was compared and contrasted. For this purpose, “Classic” and “Mini” columns were evaluated, having diameters of Xmm and Ymm, respectively.

Samples containing aflatoxin at the ppb level and deoxynivalenol at the ppm level were analyzed, both in the form of spiked buffer solutions and in naturally contaminated Reference Materials, using each column under standard protocol conditions.

In all cases evaluated, it was observed that the Mini column with a narrower diameter provided superior performance over the standard Classic column. Gel bed volume comparisons at 1:1, 2:3 and 1:3 were made, with recovery rates for the Mini remaining higher independent of gel bed volume.

Advanced synthesis of mycotoxin sulfates

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During phase II detoxification mycotoxin sulfates are formed, which are usually not detected using standard protocols and quantification is not possible due to lack of reference materials. Applying standard protocols using different sulfur trioxide complexes (e.g. $SO_3 \cdot NEt_3$) the synthesis of zearalenone sulfates was not successful. A new method for the synthesis of phenolic sulfates was published by Scott D. Taylor et al. in 2004 [1] and we decided to use this procedure for the first synthetic preparation of different mycotoxin sulfates.
First chlorosulfonic acid 2,2,2-trichloroethyl ester was prepared and different protected sulfates of mycotoxin mimics for alternariol and zearalenone were synthesized (fig. 1).

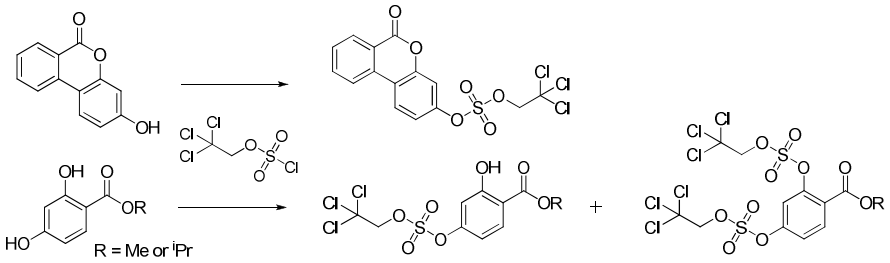

Figure 1: Synthesis of protected sulfates

Deprotection of the 2,2,2-trichloroethyl group using palladium catalyzed transfer hydrogenation with ammonium formate was successful and led to the ammonium salts of the corresponding sulfates (fig. 2).

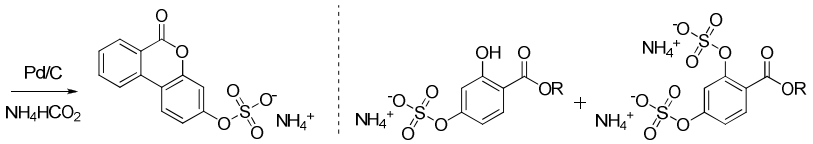

Figure 2: Palladium catalyzed transfer hydrogenation

Furthermore AOH-3-sulfate was synthesized starting from alternariol-7,9-dibenzyl ether (ADBE), using a modified hydrogenation protocol (fig. 3).

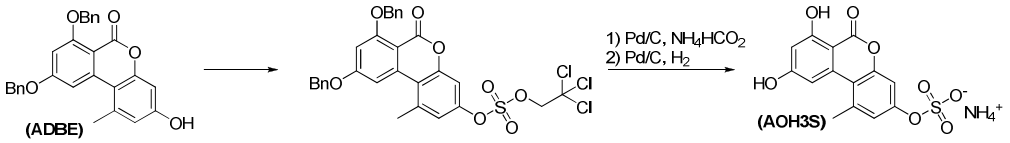

Figure 3: Synthesis of alternariol-3-sulfate (AOH3S)

The synthesis of ZON-sulfates using zinc instead of palladium on activated carbon during the final deprotection, to avoid hydrogenation of the ZON double bond, is currently under investigation.

Reference

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Analysis of ochratoxin A using online-SPE coupled with HPLC-fluorescence

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Online SPE processing is becoming increasingly popular for automated analysis, due to the improvement of laboratory efficiency, enabling fast and effective clean-up and concentration of the target compound.
Ochratoxin A is a toxic and carcinogenic fungal secondary metabolite. It is a potent nephrotoxin, carcinogen and teratogen. Hence European Union legislative bodies are imposing low Ochratoxin A limits on many affected matrices. Very sensitive methods are necessary to meet the official food control requirements. Therefore the standard reference procedures are based on immunoaffinity column clean-up, coupled to LC with fluorescence detection. Immunoaffinity column clean-up is widespread in mycotoxin analysis due to its high sensitivity and selectivity. It is, however, the most critical and time consuming step in the process. Commercial laboratories are challenged to manage an increasing amount of samples with unvarying precision and sensitivity. Approaches based on extraction and measurement with LC-MS/MS is for many matrices not applicable. Hence high-speed analysis based on immunoaffinity SPE cartridges coupled to LC and fluorescence detection represents an optimized solution for these competing requirements. Fully automated sample application and clean-up by immunoaffinity SPE, and subsequent LC-FD analysis can be coupled to obtain runtimes of 10' per sample. The Clean-up steps and HPLC-runs are performed simultaneously. The application of the developed method shall meet the criteria of the European legislation and show the same performance characteristics as obtained by the reference method.

References

DIN EN 15829:2010: Determination of ochratoxin A in currants, raisins, sultanas, mixed dried fruit and dried figs.

DIN EN 14132:2003: Determination of ochratoxin A in barley and roasted coffee.

Commission Regulation (EC) No 401/2006.

Development of multi-fusariotoxin reference materials: Tools for emerging mycotoxin analysis and *in vivo* experiments

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Preparation of reference materials such as compound feed with well-known levels of contaminants is of paramount importance in research projects dealing with the fate and the transfer of contaminants in the food chain. Moreover, the development of new analytical methods for less known toxins (emerging mycotoxins) enables monitoring of food and feed quality. Most of the existing reference materials have emphasized on "traditional" mycotoxins, such as aflatoxins, ochratoxin A, fumonisins, patulin and some trichothecenes. However, one of the most common grain contaminating genus of fungi, *Fusarium* spp., is also capable of producing several toxic secondary metabolites including the so-called emerging mycotoxins such as fusaproliferin, beauvericin, enniatins. So far, no reference material is available for these metabolites due to their late recognition and understanding of their role as mycotoxins. The Belgian National Reference Laboratory therefore prepared cocktail reference materials. The new material combined regulated mycotoxins (deoxynivalenol, zearalenone) and non-regulated toxins (T2-toxin, HT2-toxin, enniatins, beauvericin) in single filling wheat flour. Testing and confirmation of sufficient homogeneity as well as a stability study were carried out according to the relevant protocols [Fearn and Thompson, 2001, Thompson et al. 2006 IUPAC Technical Report & ISO Guide 35:2006]. The proficiency test for assigning the mass fraction values for the toxins is ongoing.

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Determination of deoxynivalenol, zearalenone, ochratoxin A and their metabolites in physiological samples of salmon with liquid chromatography – tandem mass spectrometry

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Cultured fish can be exposed to mycotoxins through contaminated feeds. Only limited knowledge on the transfer of known mycotoxins and their metabolites into edible tissue of fish is available so far. In cooperation with Skretting Aquaculture Research, Stavanger, Norway, studies on the effects of deoxynivalenol (DON), zearalenone (ZON) and ochratoxin A (OTA) on cultured salmon were conducted which also included the investigation of the carry-over of these toxins (Döll et al., 2011). For that reason, LC-MS/MS methods for the determination of these toxins and their known metabolites in physiological samples of fish were developed.

Blood plasma and freeze-dried fish filet were incubated with beta-glucuronidase before extraction to account for possible toxin conjugates. For the analysis of DON and de-epoxy-DON the samples were extracted and cleaned-up with immunoaffinity columns (IAC) according to Valenta et al. (2003) with slight modifications. For the determination of ZON and the metabolites alpha-zearalenol, beta-zearalenol, zearalanone, alpha-zearalanol and beta-zearalanol, a sample preparation method based on the DON method using IAC's specific for ZON has been developed. For the determination of OTA and ochratoxin alpha (OT-alpha), the samples were extracted with chloroform after acidification and cleaned up with liquid-liquid-partition. The toxins were determined with HPLC coupled to a triple quadrupole mass spectrometer equipped with an electrospray ionisation source (LC-ESI-MS/MS) which was operated in negative mode.

The detection limits of DON/de-epoxy-DON were 0.4/0.8 ng/ml in plasma and 0.7/1.5 ng/g in freeze-dried fish filet. The recovery was 58/65% in case of plasma and 77/80% in case of fish filet. The detection limits of ZON and its metabolites were in the range of 0.1-0.3 ng/ml and g, respectively, for both plasma and freeze-dried fish filet. The recovery amounted to 60-76% (plasma) and 83-90% (freeze-dried fish filet), respectively. In case of OTA/OT-alpha, the detection limits for both matrices were 0.1/0.2 ng/ml and g, respectively, the recovery amounted to 31/50% (plasma) and 25/48% (freeze-dried fish filet).

The LC-MS/MS methods for DON, ZON and metabolites are well suited for fish matrices as they are very sensitive and the recovery is acceptable. In contrast, the recovery of OTA and OT-alpha is too low, probably because of matrix effects.
Therefore, additional clean-up with IAC's or the use of internal standards should be tested.

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An UHPLC-MS/MS multi-mycotoxin method for maize silage

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Seen the increasing use of maize silage and the negative effect of mycotoxins on animal production and welfare, the presence of these secondary metabolites can lead to important economic losses and veterinary costs. Cases of reproductive and immune status problems as well as reduced feed intake/ production have been reported [1-2]. For maize silage, the most prevalent postharvest mould genera are *Penicillium*, *Aspergillus*, *Byssochlamys* and *Monascus*. In addition, *Fusarium* species can occur at the preharvest stage. As a result, both pre- and postharvest mycotoxins such as fumonisins, AFB1, citrinin, gliotoxin, ZEN, DON, roquefortine C, mycophenolic acid, patulin and others have been detected in maize silage [3-7]. The presence of a range of mycotoxins implies that synergetic, additive or antagonistic effects can occur. This emphasizes the need for developing multi-mycotoxin methods allowing to detect several mycotoxins in one analytical run.

The aim of this study was to develop and validate a multi-mycotoxin UHPLC-MS/MS method for maize silage. The mass spectrometric parameters were optimized on the Xevo™ TQ mass spectrometer (Waters), whereas the chromatographic conditions were optimized on the Acquity™ UPLC (Waters) system using an UPLC BEH C_{18} column (2.1x100 mm, 1.7 µm). The extraction was performed using MeOH and ACN/H_2O (84/16, v/v), followed by purification on a Waters Oasis™ HLB column. The final optimized method was validated according to Commission Decision 2005/657/EC. Performance characteristics such as specificity, linearity, possible matrix effects, recovery (R_A), repeatability (RSD_r), reproducibility (RSD_R) and the limit of detection were evaluated. While the R_A ranged between 71% and 115%, RSD_r and RSD_R varied between [5-39%] and [7-42%], respectively. This method was successfully applied for quantifying mycotoxins present in maize silage samples. The results indicated that mainly *Fusarium* toxins such as DON, ZEN, fumonisins, fusaric acid, enniatins and beauvericin were present in the samples examined. As for the postharvest mycotoxins, roquefortine C and mycophenolic acid seemed to be the main contaminants. The occurrence of the latter mycotoxins could be correlated to the presence of *P. roqueforti/paneum* in these commodities.

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Biomarkers of exposure to deoxynivalenol and zearalenone: Method development and its application to human urine samples

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To estimate human exposure to a certain mycotoxin at an individual level it is essential to perform an accurate risk assessment based on the determination of suitable biomarkers. So far deoxynivalenol (DON) and zearalenone (ZEN), exposure was estimated from dietary average intakes or by measurement of the native toxin in urine after enzymatic hydrolysis with β-glucuronidase (Meky et al 2003, Metzler et al 2010).

We report an LC-MS/MS based method for the direct quantification of the *Fusarium* toxins DON and ZEN, their glucuronide conjugates as well as alpha- and beta-zearalenol in human urine. The developed method is based on a simple, fast and inexpensive "dilute and shoot" approach where sample cleanup is minimized to a centrifugation and dilution step.

Challenges in the method development like severe matrix effects, matrix interferences, and the optimization of signal intensities are discussed. The method was applied to urine samples obtained from Austrian volunteers consuming regular and cereal restricted diet. Results are discussed in the light of exposure assessment and the established limits of tolerated daily intake (SCF 2002).

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Determination of T-2 toxin and its metabolites in physiological samples with LC-MS/MS after sample preparation with QuEChERS

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T-2 toxin, produced by various *Fusarium* fungi, is one of the most toxic mycotoxins among the trichothecenes. For studies on kinetics, metabolism and carry-over of T-2 toxin selective and sensitive analytical methods are needed. However, such methods including time efficient and economic sample preparation for physiological samples like blood, bile, tissue and excreta are limited. Therefore the aim was to develop a selective and sensitive LC-MS/MS method for the determination of T-2 toxin and its metabolites HT-2 toxin, neosolaniol, T-2 triol and T-2 tetraol to enable metabolic studies in laying hens.

For the development of sample preparation methods several materials like Chem Elut™ cartridges (Varian), Bond Elut Mycotoxin™ cartridges (Varian) but also QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) based functionalized sorbents with primary and secondary amines (PSA) were tested for their suitability to extract and clean up blood, bile and excreta. Additionally different beta-glucuronidases (helix pomatia, patella vulgate and bovine liver) were tested for their suitability to hydrolyse glucuronides. The method development was carried out with and without d3-T-2 toxin as internal standard.
For LC-MS/MS measurements a 4000 Qtrap (Applied Biosystems) in positive ESI (electrospray ionization) mode coupled with an HPLC (1200 Series, Agilent) was used. Several experiments were carried out to optimize the mass spectrometric and chromatographic conditions. T-2 toxin and its metabolites were separated using gradient elution on a Pursuit™ XRs Ultra 2.8 column (Varian) with the eluents 0.13 mM ammonium acetate (A) and methanol (B) at a start composition of 93/7 (A/B, v/v).

For incubation, beta-glucuronidase from bovine liver (6000 U/ml, Sigma Aldrich) was selected as only this glucuronidase among the enzyme preparations tested did not degrade T-2 toxin during the incubation. First results of the development of the sample preparation methods showed that the QuEChERS material used according to a method of Plössel et al. (2006) with slight modifications is suitable for cleaning up blood plasma and bile. First results for excreta using this material showed satisfactory results as well, but further experiments have to be conducted to improve the clean up procedure.

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SensiTOX: Sensitive mycotoxin analysis by modulated fluorescence polarisation

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In the context of the project SensiTOX an analysis system is developed for the detection of mycotoxins in cereals based on fluorescence polarisation. This system is to be applied primarily in the range of the inspection of incoming goods e.g. of mills. To that it has to be fast, robust, quantitative and easy to handle so that unskilled laboratory employees can achieve reliable results as well.
For this purpose the fluorescence polarisation has proved itself as fast and reliable method. In comparative measurements with established procedures the fluorescence polarisation shows a comparable accuracy to HPLC with different detectors (e.g. UV, fluorescence and mass spectrometer). In addition to the advantages of the conventional fluorescence polarisation the project SensiTOX realizes an increase of the sensitivity and an increment of the sample throughput. The increase of the sensitivity is implemented by different modulations of the excitation light and the increment of the sample throughput by transfer of the fluorescence polarisation to microtiter plate format. Thereby mycotoxins can be determined in a reliable and quantitative way without conducting a higher concentration. Mycotoxins often occur only in very limited concentrations and their critical values are very low (e.g. aflatoxin and ochratoxin A). At the same time the analysis duration of the method is to be kept low, to be applied in an effective way as rapid method besides procedures like DipStick.

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Production of auxin from tryptamine: A role in virulence of *Fusarium graminearum*?

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The genome wide analysis of gene expression of *Fusarium graminearum* infected barley revealed that genes involved in the synthesis of tryptophan from chorismate are upregulated [1]. Co-regulated with the tryptophan biosynthetic genes is a gene predicted to encode an aromatic-L-amino-acid decarboxylase, a putative tryptophan decarboxylase. The product of this reaction, tryptamine, and its derivative 5-hydroxy-tryptophan, were reported to play an important role in rice in the peroxidase-mediated synthesis of an antifungal cell wall barrier [2].
We have developed a LC-MS/MS method for tryptophane and derivatives and we found that *F. graminearum* in axenic culture is able to efficiently metabolize tryptamine (but not tryptophan) into the plant hormone indole-3-acetic acid (commonly known as auxin). Our hypothesis is that the fungal pathogen can divert tryptamine away from the cell wall mediated defense pathway and convert it into a metabolite increasing plant susceptibility by shifting resources from defense to plant growth [3]. We have searched the *F. graminearum* genome sequence for putative auxin biosynthesis genes, in order to generate gene disruption mutants to be able to experimentally test the effect of candidate genes on auxin production and virulence.

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Infection process and diversity of *Fusarium* species on maize in the UK and their contribution to mycotoxin contamination

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Fusarium spp. and their mycotoxins both pose a threat to the expanding maize area in the UK and to small grain cereals. The diversity of mycotoxin producing *Fusarium* spp. in the United Kingdom on maize has not been profiled since the late 1970s (Cook, 1978). A three year incidence study is planned for 2011-2013 of *Fusarium* spp. on maize and will be used to build a profile of predominant and emerging *Fusarium* spp. and their associated mycotoxins within maize growing regions in the UK. An initial incidence study in 2010 from five UK sites including grain and forage maize showed that the most frequently isolated species were *F. graminearum* and *F. culmorum*; *F. poae*, *F. verticilliodes*, *F. solani*, *F. crookwellense*, and *F. subglutinans* were also present. *F. graminearum* and *F. culmorum* isolates were positively identified to be deoxynivalenol producing strains based on the tri5 gene (Bakan et al, 2002). Studies on the resistance of a diverse set of maize cultivars to mycotoxin producing species are being implemented in field and growth room studies in 2011 and 2012. A preliminary resistance study indicated that one maize cultivar had a significantly higher emergence than two others when inoculated as seedlings with *F. graminearum* and *F. culmorum*. Further studies will focus on infection processes in maize and potential mycotoxin accumulation.

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In vitro models for studying the fumonisin masking mechanism in maize

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Hidden fumonisins are fumonisin derivatives which can be covalently or not covalently bound with food macroconstituents. Such derivatives can co-exist together with native forms, both in raw maize and in maize based products and are detectable only through the application of a hydrolysis step on the matrix [1]. In particular, the most intriguing aspect of this phenomenon is that the nature of matrix-fumonisin interactions is still unravelled. To elucidate this fact, there are two main hypotheses, being the former based on the formation of covalently bound fumonisins derivatives and the latter on the formation of an associative complexation between native forms and macromolecules such as proteins or starch. In this study some *in vitro* experiments were carried out with the aim to obtain more data about the nature of this masking mechanism. As first, an *in vitro* digestion model [2] has been applied to several raw maize samples in order to study hidden fumonisins bioaccessibility after gastrointestinal digestion. Then, the stability under gastrointestinal conditions of two chemically synthetised covalent fumonisin adducts (Lysil-fumonisin B1 and Fructosyl-fumonisin B1) has been studied throughout the application of the same protocol, to evaluate the possible release of the free form upon enzymatic hydrolysis. Moreover, the conventional fumonisin extraction procedure from raw maize was compared to a QuEChERS-like approach [3]: in the latter case, in deed, the application of a salting out step could induce the release of hidden forms due to protein precipitation, supporting thus the complexation hypothesis. Finally, titration experiments were performed among fumonisin B1 and zein or starch, respectively, in order to understand in which conditions the masking phenomenon takes place and what types of interaction occur between fumonisins and these compounds. All the experiments here presented were based on *in vitro* model systems, providing thus a clearer idea about the masking mechanisms.

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Monascus purpureus: Characterization of two new metabolites from red yeast rice

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Red yeast rice (angkak, koji) is produced by the fermentation of rice with the fungus *Monascus purpureus*. Secondary metabolites generated during growth are responsible for its use as food colorant and preservative in Asian countries as well as in Chinese folk medicine. Several metabolites such as six major pigments, citrinin and pharmacologically active monacolin K have been known for decades. In recent years, a variety of new metabolites were described in literature whereas information concerning the biological activity is rare or incomplete. Due to the increasing interest in red yeast rice products in Western countries based on its natural origin, it is important to characterize the occurring secondary metabolites in order to evaluate the safety of these products. Therefore, we developed a method to isolate *Monascus* constituents and elucidated the structure of two so far unknown metabolites.

M. purpureus was cultivated on rice in petri dishes at 30°C. After 3 weeks, the cultures were dried, pulverized and extracted with methanol. The extract was enriched on a C_{18} cartriged and the fraction eluting with 80% methanol was further analyzed. Preparative HPLC-UV was utilized for the isolation of ten known metabolites that were identified by their UV-spectra and exact masses. In addition, two new metabolites of red color were isolated. By means of FTMS as well as 1D- and 2D- NMR experiments, their structures were elucidated. From the data, the molecular formulas $C_{23}H_{31}NO_4$ and $C_{21}H_{27}NO_4$ were derived. The difference of C_2H_4 reflects the characteristics of *Monascus* metabolites to occur pairwise implying a C_7H_{15} and C_5H_{11} side chain. Furthermore, the existence of a methyl ester moiety in the new metabolites was shown to be an artifact due to the use of methanol in the isolation procedure. The methyl ester did not occur in acetonitrile extracts whereas in ethanol extracts, only the ethyl ester was detectable. Examination of the fragmentation pattern of the newly identified substances confirmed these findings.

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Paradox results between mycotoxins and cytotoxicity in selected *Aspergillus niger* species

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The genus *Aspergillus* is one of the most common and widely distributed fungi in the world. Many species of *Aspergillus* play an important role in the biotech industry, agriculture, as well as in public health. Filamentous fungi in the *Aspergillus* section *Nigri* (the black aspergilli) are some of the most important mycotoxigenic food and feed contaminants.
Fifteen *Aspergillus* sect. *Nigri* strains (ACBR, CBS) were used in this study. All fungal strains were grown for one week at 25°C on malt extract agar. For the extraction, a mixture of acetonitrile, water and acetic acid (79/20/1, v/v/v) was used. The MTT-cell culture assay was used to determine cytotoxicity of potential medicinal agent and other toxic materials. Additionally, extracts were tested for mycotoxin-production using of LC-ESI-MS/MS-based multimycotoxin method. This multi-mycotoxin method is able to identify quantify 247 mycotoxins.
Two strains, *Aspergillus vodensis* (MA5490) and *Aspergillus tubingensis* (MA5482) showed no cytotoxic effect. Six strains, *Aspergillus awamori* (MA5486), *Aspergillus brasiliensis* (MA5491), *Aspergillus costariaeuis* (MA5492), *Aspergillus eucalypticola* (MA5489), *Aspergillus foetidus* (MA5488), and *Aspergillus niger* (MA5183) were low cytotoxic. *Aspergillus awamori* (MA5480), *Aspergillus costeariaeuis* (MA5487), *Aspergillus neoniger* (MA5484), *Aspergillus niger* (MA5481), *Aspergillus piperis* (MA5479), and *Aspergillus tubingensis* (MA5493) were medium cytotoxic. Only *Aspergillus neoniger* (MA5484) was highly cytotoxic in the MTT-assay and produced pyranonigrin A (28067 µg/l) and aspergillimide (1,2 µg/l). *Aspergillus costariaeuis* (MA5492) produced ochratoxin A (2040 µg/l), ochratoxin B (814 µg/l) ochratoxin alpha (1520 µg/l), aspergillimide (0,874 µg/l), pyranonigin A (1153 µg/l) and was low cytotoxic. In contrast, *Aspergillus costariaeuis* (MA5487) produced malformin C (8360 µg/l) and xanthomegnin (13,7 µg/l). *Aspergillus tubingensis* (MA5493) produced fumonisin B2 (664 µg/l), fumonisin B4 (9130 µg/l), pyranonigrin A (3155 µg/l), aspergillimide (0,6 µg/l).

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Air humidity and soil moisture control within-field variations of mycotoxin contamination in wheat

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Humidity is an important determinant of the mycotoxin production (DON, ZEA) by *Fusarium* species in the grain ears. From a landscape perspective humidity is not evenly distributed across fields. The topographically-controlled redistribution of water within a single field rather leads to spatially heterogeneous soil water content and air humidity. Therefore we hypothesized that these terrain-related differences control the spatial distribution of mycotoxins. We demonstrated [1] how topographic relief differences of a major process-related variable, i.e. canopy air humidity during flowering and grain filling, are related to mycotoxin concentrations and [2] whether soil redox status can be used as a qualitative indicator for soil moisture and mycotoxin distribution at the field scale.

To test this hypothesis we studied the mycotoxin concentrations at contrasting relief positions, i.e. hilltops and depressions characterized by soils of different soil moisture regimes, on ten winter wheat fields in 2006 and 2007. Maize was the preceding crop and minimum tillage was practiced at the fields.

The different topographic positions were associated with moderate differences in DON and ZEA concentrations in 2006, but with significant differences in 2007: Up to six times higher median values of mycotoxins were detected in the depressions. This corresponds to a higher air humidity as well as redoximorphic properties in soil profiles, which confirms our hypothesis at least for years showing wetter conditions in sensitive time windows for *Fusarium* infections. The results support the interpretation that the increased water availability in the topographic depression positions – coming from soil moisture and/or air humidity – and the possibility for an intensified spore dispersion and a successful colonisation and longer growth period of the fusaria in the ears of wheat, together provide an opportunity for increased DON and ZEA accumulation.

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The comparison of winter rye and winter triticale mycotoxin contaminations

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Quality estimation of winter rye and winter triticale yield in aspect of mycotoxins content is very important regarding consumer health. Mycotoxins produced by fungi are the one of the most dangerous compounds which contaminate cereal products. Most frequent mycotoxins occuring in cereals are trichothecenes produced by *Fusarium* spp. but also aflatoxins and ochratoxins produced by the genera *Penicilium* and *Aspergillus*.
The aim of this study was determination of mycotoxins content in different cultivars of winter rye and winter triticale by use of the Enzyme-Linked ImmunoSorbent Assay (ELISA) method.
The mycotoxin contamination of cultivars winter rye and triticale was different. Triticale cultivars were more contaminated by toxin T-2, cultivars of winter rye by deoxynivalenol. In several cultivars of winter rye deoxynivalenol content far exceeded the maximum permissible levels established for this compound in cereal grains by the EU. Aflatoxins and ochratoxins were not found in grain of tested cultivars.
Amongst tested cultivars of both cereal species were mycotoxins free, and only such cultivars should be recommended to practice what would be create the opportunity of significant reduction of mycotoxins content in cereals.

Keywords: winter rye, winter triticale, mycotoxins, ELISA

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The use of differential in gel electrophoresis (DIGE) to study proteome of *Aspergillus ochraceus* under various conditions

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Aspergillus ochraceus is a filamentous fungus, which produces secondary metabolites called mycotoxins (ochratoxin A, and ochratoxin B etc.). Ochratoxin A (OTA) is widely distributed in Europe and North America, and has been found as a natural contaminant in agricultural commodities. OTA is an effective nephrotoxin to several animals and has been related with the human kidney disease known as Balkan Endemic Nephropathy. The aim of the study was to compare the proteome of *A. ochraceus* cultivated in two different media by using the DIGE technique. *Aspergillus ochraceus* NRRL 5221 was cultivated in submerged culture (malt extract broth and yeast extract sucrose broth) at 25°C with shaking at 150 rpm for 20 days in the dark. After harvesting the mycelium, cell lysis was performed by grinding it in liquid nitrogen. Proteins from mycelia were extracted using lysis buffer and precipitated with TCA-acetone. The re-dissolved proteins were separated by differential gel electrophoresis. We evaluated 790 spots from the gels. Fifty eight spots (7.3%) were downregulated, while eighty two spots (10.4%) were upregulated in *A. ochraceus* NRRL 5221 when cultivated in YES (Yeast extract sucrose) broth. All these changes are at least 5 fold. 650 spots (93.6%) were common in both. This study showed that cultivation media definitely have an effect on the protein expression of *Aspergillus ochraceus*.

De-acetylation of 3-ADON and 15-ADON by plant and *Fusarium* enzymes

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Deoxynivalenol producing *Fusarium graminearum* isolates can be subdivided based on the metabolite that initially predominates in axenic cultures into 3 acetyl-DON (3-ADON) or 15-acetyl-DON (15-ADON) chemotype. The acetylation can dramatically change the water solubility and membrane permeability of the DON derivatives and the interaction with targets and detoxficiation enzymes. Using a wheat germ coupled *in vitro* transcription and translation system we have determined that the order of toxicity for wheat ribosomes is 15 ADON 〉 DON 〉〉 3-ADON. It is unclear what caused the recent massive chemotype shifts of *F. graminearum* observed in North American [1] and other regions. Both types of strains initially produce 3,15-diacetyl-DON, and the rates of the deacetylation by different allelic variants of the Tri8 esterase determines which intermediate is accumulated [2]. It was proposed that the conversion of 3 DON and 15-ADON into the final product DON is mediated by carboxylesterases other than Tri8 [2].
Using heterologous expression in yeast we could show that the *TRI8* gene product of the 15-ADON strain *Fusarium* PH-1 is an esterase that also removes the acetyl group from 3-ADON and converts it into the more toxic DON. Yeast transformants of a host strain with a deletion of the yeast acetyltransferase gene *AYT1* showed increased 3 ADON sensitivity upon expression of *FgTRI8* compared to transformants containing the empty vector. We have also expressed a predicted *TRI8* paralog (FGSG_03846) in yeast, but did not observe esterase activity with 15 or 3-ADON in the transformants.
Deacetylation reactions are most likely highly relevant in the early stage of the plant pathogen interaction and are not only catalyzed by fungal enzymes. We characterized this reaction in *Arabidopsis thaliana*, the monocot model system *Brachypodium distachyon*, and wheat. Especially wheat ears can efficiently deacetylate 3 ADON into the more toxic DON. In order to identify the relevant member (33 genes), we started to characterize the carboxylesterase family encoded by the *Brachypodium* genome.

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NaCl dependent regulation of ochratoxin A biosynthesis in *Penicillium*

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P. nordicum is an ochratoxin A producing filamentous fungi which occurs mainly on NaCl rich habitats like cheeses or dry cured meats. *P. verrucosum* is a frequent contaminant of cereals but also of brined olives. It could be shown that sodium chloride has a profound influence on the regulation of ochratoxin A biosynthesis in both *Penicillium* species. High amounts of ochratoxin A are produced by *P. nordicum* over a wide concentration range of NaCl (5 – 100 g/l) with an optimum at about 20 g/l when growing on YES medium. The biosynthesis of ochratoxin A at high NaCl concentrations (100 g/l) is delayed, but also reaches high amounts after a prolonged incubation phase. Elevated concentrations of NaCl lead to hyperosmotic stress conditions which are detected by a sensor kinase. The signal is then transduced by the high osmolarity glycerol (*hog1*) MAP kinase pathway, resulting in several intracellular adaptations. The induction of the *hog1* signaling pathway is detectable by measuring the phosphorylation of the HOG1 protein by western blot and leads to an increased ochratoxin A biosynthesis which ensures a chloride homeostasis in the fungal cell even under high NaCl conditions through excretion of the chloride containing OTA molecule out of the cell. The higher the salt concentration, the more phosphorylated HOG1 can be detected. This mechanism obviously increases the competitiveness of ochratoxin A producing Penicillia in NaCl rich media. *P. verrucosum* shifts secondary metabolite biosynthesis from citrinin at low to ochratoxin at higher NaCl concentrations. Cereals, the habitat of *P. verrucosum*, are less salt-rich than e.g. fermented meat. In fact, they are typically exposed to sunlight what necessitates also a protectant against short wave daylight. In this case, citrinin could serve as a light-protective secondary metabolite.

Influence of *Fusarium* diseases on yield and quality of *Zea mays*

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Introduction: The economic importance of maize has increased in Germany in the last years. In this study different fungicide treatments were tested for their effect on mycotoxin concentration in maize silage, Corn Cob Mix and kernels to investigate the influence of *Fusarium* diseases on yield and quality.
Furthermore the infestation with *Fusarium* spp. was observed within cropping systems. Different crop rotation and tillage systems as well as different rates of nitrogen were examined.

Material and Methods: Field trials were set up at two locations in Germany. The first location was in Schleswig-Holstein with a maritime affected climate. The other location was chosen in Bavaria with a continental affected climate to show a differentiated disease progression of *Fusarium* spp.. The cropping system trial was only located in Schleswig-Holstein.
The susceptible cultivar ´Lorado´ was used for the field trials. The plots were treated with azols in different development stages. In order to interpret the experiment, maize silage, Corn-Cob-Mix and kernels were analyzed by molecular methods (PCR, qPCR) and HPLC or LC/MS. In addition the quality of forage was evaluated (Weender Analysis).
Regarding the cropping systems seven different crop rotations with up to four nitrogen rates were investigated for their influence on *Fusarium* disease severity.

Results and Discussion: Qualitative and quantitative PCR-analytics showed a species shifting inside the *Fusarium* spectrum. It is suggested that this was induced by fungicide applications. However, no effect on the amount of mycotoxins was detected. An influence of the fungicide treatments on the yield could be measured. In further experiments it has to be tested to which extent other fungal pathogens caused an infestation. There were no correlations between forage quality, mycotoxin quantity and yield.
First results indicate effects of crop rotation, nitrogen supply and tillage treatments on *Fusarium* infestation.

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Fusarium head blight resistance of winter triticale breeding lines and accumulation of ergolsterol and *Fusarium* mycotoxins in grain

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Resistance of 25 breeding lines of winter triticale was evaluated. Lines were sown in two field experiments located in Cerekwica, Western Poland and in Radzikow, Central Poland. Triticale heads were inoculated at flowering stage with conidial suspension of *Fusarium culmorum*. Three isolates producing deoxynivalenol (DON) and nivalenol (NIV) were applied. *Fusarium* head blight was scored and number of *Fusarium* damaged kernels (FDK) was determined. Grain was analyzed for concentration of *Fusarium* metabolites (ERG, DON, NIV) using HPLC technique.

Average FHB severity in Radzikow was lower than in Cerekwica. FHB ranged from 0 to 13.3% in Radzikow and from 2.0 to 26.8% in Cerekwica. Average FDK was 10-fold higher in Cerekwica (18.1%) than in Radzikow (1.6%). FDK ranged from 7.3 to 51.5% in Cerekwica and from 0.1 to 4.9% in Radzikow.

Chromatographic analysis revealed presence of DON and NIV in triticale grain. Average amount of DON in grain in Radzikow was 0.92 ppm and was 3-fold lower than in Cerekwica (2.76 ppm). Similarly, variation range was wider in Cerekwica (0.40-11.49 ppm) than in Radzikow (0.25-2.98 ppm). DON concentration in grain was relatively high, taking into consideration low proportion of *Fusarium*-damaged kernels. Amount of DON in triticale grain samples was higher than NIV amount (about 3-fold in Radzikow and 17-fold in Cerekwica). Average NIV concentration was 0.37 ppm in Radzikow, at a range 0 - 1.62 ppm, and 0.18 in Cerekwica, at a range 0 – 0.56 ppm.

FDK correlated significantly with DON amount for all samples as well as for locations separately. As regards NIV concentration we did not found significant correlation with FDK for the whole experiment. However, in both locations linear relationship of these characters was observed and was significant for Cerekwica.

On the basis the results obtained in two environments triticale lines were ranked according to the 4 resistance types. Triticale lines ‘DAD 172’ and ‘DAD 305’ had the highest resistance of all types. Five lines:‘LAD 1167’, ‘DAD 238’, ‘SZD 1063’, ‘DAD 263’ and ‘CHD 2197’ had medium resistance of type 1+2 or 3 (head and kernel infection) but were highly resistant to mycotoxin accumulation (type 4). Line SZDR 741was resistant to head and kernel infection but accumulated more DON and NIV than other lines of similar resistance. The most susceptible (for all resistance types) were lines ‘BOHD 98-1’, ‘BOHT 236 F1’, ‘CHD 2220’ and cultivar ‘Grenado’. Lines ‘BOHD 142/2’ and ‘SZD 1114’ and cultivar ‘Moderato’ showed low head infection, however accompanied by high kernel damage and mycotoxin concentration. Line CHD 662, despite high FDK value, accumulated relatively low amounts of mycotoxins. The highest head infection and kernel damaged showed line LAD 1132, however it resulted in only moderate mycotoxin accumulation.

Aflatoxins in nuts and nut products from third countries and from the Bavarian market

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According to Commission Regulation (EC) No 1152/2009 the Bavarian Health and Food Safety Authority analysed 407 consignments of nuts and nut products, which were taken at Bavarian points of import in 2010. Furthermore a focal point was set on the control of nuts from market stalls and folk festivals. For this focal point samples of almonds and peanuts (n=94) have been drawn.

The samples were analysed with HPLC and fluorescence detection (CoBrA-Cell®) after extraction with acetonitrile/water and a clean-up procedure with immunoaffinity-columns.

Fourteen percent of 90 consignments of pistachios and derived products contained aflatoxin B/G concentrations above the legal limits. The maximum detected concentrations were 594 µg/kg for aflatoxin B1 and 665 µg/kg for the sum of aflatoxin B/G, respectively. For 282 consignments of hazelnuts and derived products, the rate of non-complying samples was 1.4%, with a maximum value of 25 µg/kg for aflatoxin B1 and 28 µg/kg for the sum of aflatoxin B/G, respectively. Aflatoxin-contents of 4 consignments of peanuts originating from China and 31 consignments of almonds from the United States of America were below the legal limits.
In one sample of processed peanuts (“Couli Couli” - fried peanut pulp as a pencil-thin roll) originating from Togo, which was taken during an import procedure at the Airport Munich, 801 µg/kg for the sum of aflatoxin B/G was found.

In three of 66 almond samples from market stalls and folk festivals occurred aflatoxin-contents of 21 µg/kg, 41 µg/kg, and 46 µg/kg (sum of aflatoxin B/G), respectively. In one of 28 peanut samples a content of 17 µg/kg for the sum of aflatoxin B/G was detected.

The results are presented and discussed with respect to possible health risk for the consumer.

References

Commission Regulation (EC) No 1152/2009 (27 November 2009) imposing special conditions governing the import of certain foodstuffs from certain third countries due to contamination risk by aflatoxins and repealing Decision 2006/504/EC.

Commission Regulation (EC) No 1881/2009 (19 December 2006) setting maximum levels for certain contaminants in foodstuffs.

Occurrence of the mycotoxin alternariol in honey

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Natural honey is produced by bees mainly from floral nectar, but may contain other ingredients such as pollen or honeydew. Honey has low water activity, and is generally regarded as safe in aspects of microbiological growth. However, the floral environment containing nectar and pollen may be infected with mycotoxinogenic fungi. These may grow on pollen, pre- or postharvest by honey bees, and contaminate the honey or a produce called “bee pollen” (syn. “bee bread”, a nutritional supplement). However, mycotoxins have never been reported as natural contaminants in bee products. Since *Alternaria* species occur most abundantly worldwide as saprophytes or parasites on plants, the study described here aimed at analysis of alternariol (AOH) in honey and bee pollen.

Highly sensitive enzyme immunoassays (EIA) were used to develop a simple analytical method for AOH in honey, which involved liquid-liquid partitioning with ethyl acetate. The honey extracts were analysed by two EIAs, based on monoclonal (mAb) or polyclonal (pAb) antibodies, respectively. The detection limit for AOH was at 0.5 ng/g in honey, and at 1 ng/g in bee bread/pollen. Recoveries (5-100 ng/g) were 90-100% in honey, and 30-280% in bee bread/pollen.

Samples of commercial honey (various countries of origin) were purchased in 2010/2011 from food shops in Hesse, bee bread/pollen was obtained via Internet order. Most honey samples analysed so far were weakly positive for AOH (0.5 - <10 µg/kg), all samples of bee bread/bee pollen were clearly AOH-positive (>10 ng/g). We therefore hypothesize that AOH is transferred via contaminated pollen into the honey. Since the mAb EIA consistently yielded higher results than the pAb EIA, confirmatory LC-MS/MS analyses of honey extracts were performed which qualitatively verified the presence of AOH. In conclusion, the results support the assumption that in addition to AOH, AOH-like compounds may also be frequently present in honey.

Fusarium species and *Fusarium* mycotoxins in grain of winter wheat in Poland in 2010

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Fusarium head blight is a disease of wheat caused by the complex of toxicogenic fungi in the genus *Fusarium*. The main species are: *F. graminearum* and *F. culmorum* producing deoxynivalenol (DON), nivalenol (NIV); *F. avenaceum* producing moniliformin; *F. poae* producing NIV; *F. langsethiae* and *F. sporotrichioides* producing T-2 and HT-2 toxins. Highly pathogenic are *F. graminearum* and *F. culmorum*, which can cause severe epidemics of *Fusarium* head blight. Other species are medium or weakly pathogenic, however, due to the wide prevalence also may cause contamination of grain with mycotoxins. Because of the diversity of *Fusarium* species inciting *Fusarium* head blight, it is important to monitor changes in *Fusarium* population on wheat.
In order to determine the presence *Fusarium* species on wheat and content of mycotoxins in wheat grain, 50 samples form 2010 harvest were collected. Samples originated from 25 experimental stations of COBORU (Research Centre for Cultivar Testing) form different regions of Poland. It was grain of 2 wheat cultivars 'Bogatka' (medium resistant) and 'Muszelka' (susceptible), the size of 1 kg. Kernels were placed on a selective medium (SFA) in order to determine the *Fusarium* occurence. Then grain was analysed for the content of *Fusarium* mycotoxins by gas chromatography technique (GC). Qualitative and quantitative determination of 6 *Fusarium* species in grain was done using real time-PCR technique [1]. *Fusarium* cultures identified on 7.8% of kernels, at a range 1.0 – 18.0%. For 'Muszelka' cultivar it was 9.0%, while for 'Bogatka' – 6.6%. Average content of DON was low and amounted to 0.096 ppm, at a range from 0.552 to 0.049 ppm. The content of NIV was very low – 0.055 ppm, at a range 0.049 – 0.071 ppm. The average content of DON for 'Bogatka' was 0.078 ppm, and 0.114 ppm for 'Muszelka'. Occurence of 5 *Fusarium* species was detected with the exception of *F. sporotrichiodies*. The highest was content of DNA of *F. graminearum* DNA (1.252 pg/ng), then *F. avenaceum* (0.259 pg/ng), *F. poae* (0.168 pg/ng) and *F. langhsethiae* (0.237 pg/ng). Very low was content of DNA of *F. culmorum* (0.055 pg/ng). The most frequently occurred *F. poae* (74% of samples) and *F. graminearum* (52%). New species *F. langsethiae* [2] was detected in 6 samples (5 from 3 locations in Northern Poland). These samples were analysed for T-2/HT-2 toxins, produced by *F. langsethiae* using ELISA assay. In all samples total concentration of both mycotoxins was below detection limit of 35 ppb.

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Trichothecenes and zearalenone in spelt wheat from ecological system of farming

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Besides einkorn wheat and emmer wheat, spelt is one of the oldest wheat sub-cultivars. Its cultivation had been given up for a long time, but it has been recently re-discovered and appreciated. The area of spelt plantations largely increased during the last 20 years, which can be attributed to quite wide interests, healthy lifestyle attitude, healthy food, and intensive development of organic farming. Spelt is valued due to its high nutritional quality, medicinal properties, and numerous agricultural benefits, among which better resistance to some fungal diseases as compared to other cereals, and in consequence lower contamination with mycotoxins, can be supposed as most important.
The aim of the study was determination of trichothecenes and zearalenone (the secondary metabolites of field fungi) in the spelt wheat grains and their products, originating from ecological agriculture practice. Material for study consisted of 42 samples (15 – raw material from 2009, 17 – raw material from 2010, 10 – products 2009) of grains, flakes, bran, flour, groat, pasta, and coffee. Mycotoxins (T-2 and HT-2 toxins, deoxynivalenol – DON, nivalenol – NIV, 3-acetyldeoxynivalenol – 3-ADON, monoacetoxyscirpenol – MAS, diacetoxyscirpenol – DAS, and zearalenone – ZEA) were determined using the HPLC-MS/MS method (3200 QTRAP). During sample preparation procedures, Bond Elut® Mycotoxin columns (Varian) were used. The following internal standards were applied: ^{13}C-DON, ^{13}C-T-2, ^{13}C-HT-2, ZAN. Among determined mycotoxins, DON was present at highest concentrations, both in raw materials (2009 – maximum 140 ppb, 2010 – maximum 124 ppb), and products (maximum 23.3 ppb). The highest level of DON was recorded in winter spelt grains (140 ppb), although the value was almost 10 times lower than the highest permissible level of DON in non-processed cereals (Commission Regulations (EC) 1881/2006 and 1126/2007). None of examined samples contained DAS. The 3-ADON was also absent in grains and products from 2009. In the case of raw materials both from 2009 and 2010, mean toxins concentrations (NIV, 3-ADON, MAS, T-2, HT-2) were close to the detection limits, while products contained only two of eight analysed toxins: ZEA and DON. ZEA was recorded in four products, with the highest concentration in spelt flakes (0.793 ppb). Also 40% of products were contaminated with DON at the highest content in spelt flour (23.3 ppb). Contaminants present in raw materials can be neutralized during grain processing, which was illustrated by here examined products: mean concentrations of determined mycotoxins were lower as compared to raw materials they were produced from. Negligible contamination of analyzed samples with mycotoxins confirms that spelt is resistant towards majority of fungal diseases. The spelt grain is protected against pathogens and environmental contamination by hermetic coat that is formed by closely adhering chaffs.

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The occurrence of fungi in ecologically grown cereals

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The organic farming finds more and more followers in Poland. At present, about 15 thousand farms involving in organic farming technology (having a certificate or being under transformation), are registered. Literature data indicate that cereal crops in organic farming system may represent a high level of mould infections – namely due to *Fusarium*. This fact can point out the occurrence of toxic trichothecenes, which is a potential threat for grains quality and for consumers. However, there is no complete information on field-origin and storage-origin fungi infecting cereal products from organic farming system, thus the present study aimed at collecting data on quantitative and qualitative composition of cereal grain micobiots from selected organic farms in Kujawy-Pomerania region.
Examined material was composed of grains of: rye (16), spelt (14), wheat (14), barley (5), cereal mixture (5), and buckwheat (2). The total fungi number was determined according to PN ISO 7954: 1999 with own modifications; the inoculation was made in three replicates.
Achieved results concerning analyzed cereal grain samples revealed diverse levels of mould contamination. Mean number of fungi [cfu/g] amounted to: buckwheat 2.5 x 105, barley – 1.6 x 105, cereal mixture – 3.3 x 104, spelt 1.0 x 104, wheat – 6.5 x 103, and rye – 6.0 x 103. The rye samples were dominated by moulds from genus *Aureobasidium* – 41%, *Penicillium* – 18%, *Alternaria* – 13%, and *Fusarium* – 7%.
Genus *Penicillium* – 27% along with *Aureobasidium* – 22% dominated in spelt samples, although *Alternaria* – 11%, *Cladosporium* – 8%, *Aspergillus* – 8%, and *Fusarium* – 8% were numerous as well. Among wheat samples, *Penicillium* prevailed – 49%, whereas other mould genera showed much lower levels, e.g. *Cladosporium* – 11%, *Aspergillus* – 7%, *Aureobasidium* – 7%, *Mucor* – 5%, or *Fusarium* – 5%. In barley samples, genus *Penicillium* – 43%, then *Fusarium* – 17%, and *Aspergillus* – 16% made up the largest shares. *Cladosporium* – 32% was the most numerous in cereal mixture samples, although *Aureobasidium* – 13%, *Penicillium* – 9%, *Fusarium* – 9%, and *Acremonium* – 8% were also remarkable. The buckwheat samples were dominated by moulds from genus *Penicillium* – 90%, with much lower level of *Fusarium* – 5%.
The study revealed great variability of moulds from genus *Fusarium* on organic grains of:
rye - *F. equiseti*, *F. solani*, *F. poae*, *F. sporotrichioides*;
spelt - *F. poae*, *F. graminearum*, *F. proliferatum*, *F. sporotrichioides*, *F. culmorum*;
wheat - *F. culmorum*, *F. poae*, *F. oxysporum*, *F. equiseti*, *F. solani*, *F. sporotrichioides*;
barley - *F. poae*, *F. culmorum* , *F. sporotrichioides*;
buckwheat - *F. poae*;
cereal mixture - *F. solani*, *F. tricinctum*.

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Food safety action plan: Targeted surveys of priority mycotoxins in domestically produced and imported foods available on the Canadian market

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The Food Safety Action Plan (FSAP) aims to modernize and enhance Canada's food safety system. As part of the FSAP enhanced surveillance initiative, targeted surveys are used to test various foods for specific hazards. The Government of Canada identified mycotoxins and imported foods as key priority areas of the FSAP. In response to these priorities, the Canadian Food Inspection Agency (CFIA) is conducting various targeted surveys of mycotoxin levels in domestically produced and imported foods.

In 2009-2010, aflatoxin (AF), ochratoxin A (OTA), and deoxynivalenol (DON) levels in selected domestically produced and imported select foods available at retail were examined. The levels of aflatoxins B1, B2, G1 and G2 were measured in 51 samples of dried figs and 49 samples of dried dates. AF levels were quantified by HPLC/fluorescence and confirmed by HPLC/MS/MS. Only 4 dried fig samples out of a total of 100 dried fig and date samples contained AF levels ranging from 7.5 to 78.7 ppb. A total of 450 samples were tested for OTA by LC/MS/MS. The commodities tested included infant formula, infant cereals, beer, wine, grape juice, wheat products, corn products and oat products. The majority of the samples (98%) did not exceed Health Canada's proposed guidelines for OTA. However, six samples of infant cereals and three samples of oat products had measured OTA levels between 0.3 ppb and 7.2 ppb, which exceeded the proposed guidelines. Grain-based products (150 samples of wheat-, corn- and oat-based products) were tested for DON by LC/MS/MS. DON was detected in 87 (58%) samples. The measured DON levels ranged from 0.01 ppm to 6.01 ppm in wheat products; 0.01 ppm to 1.38 ppm in corn products; and 0.02 ppm to 0.13 ppm in oat products. All OTA violative results and all positive results (AF, OTA and DON) were assessed to determine whether they posed any potential health risks to consumers. Appropriate follow-up action was pursued dependent on the magnitude of the health risk.

In 2010-2011, AF, fumonisins, OTA, and DON levels in selected domestically produced and imported foods available at retail are being examined. The AF survey encompasses 700 samples consisting of corn-based products, dried figs and dried dates, and nutmeats. The levels of the fumonisins B1 and B2 are slated to be measured in 300 corn-based samples. OTA and DON levels in 1000 samples (including infant formula, breakfast cereals, wheat products, corn products, oat products, wine, beer and dried fruit) have yet to be analysed. Preliminary data on mycotoxin levels in these specific commodities will be presented.

The occurrence of mycotoxins in spelt products

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Mycotoxins are secondary metabolites produced by fungi from *Aspergillus*, *Penicillium* and *Fusarium* genera, which are main producers of aflatoxins, ochratoxin A, trichothecens and zearalenon. Negative impact of the substances on human and animals health is widely proven. The most dangerous aflatoxins and ochratoxin A have been classified as carcinogenic by WHO-IARC. Detoxification of contaminated grain and food proved to be very difficult, complicated and frequently ineffective.
Rising significance of lifestyle chronic diseases caused by nutrition errors force involvement of pro-health food. High quality grain products are very important element of well-balanced, healthy diet and main food products throughout the world. Spelt and its products are very important elements of nowadays healthy diet. Spelt was grown from ancient times but now it is renewed in organic farming in Europe and North America mainly due to the nutritional value.
The aim of this study was to determine level of mycotoxins in spelt products available on the polish market. In those samples the occurrence of mycotoxins was tested. Spelt products were collected in 2009 and 2010. The Enzyme-Linked ImmunoSorbent Assay (ELISA) method was used to identify aflatoxins, ochratoxin A, T-2 toxin, deoxynivalenol and zearalenone.
In all samples from 2009 and 2010 T-2 toxin was found and identified in amount below 75 µg/kg. In samples collected in 2009 ochratoxin A and zearalenon was not found. There was one sample with aflatoxins and eleven with deoxynivalenol. In all examined samples tolerated level of mycotoxins was not exceeded. In samples from 2010 in four aflatoxins were found. In two of them tolerated level of mycotoxin was exceeded. In one sample zearalenon was detected in amount 45,2 µg/kg, two samples contained deoxynivalenol (34,3 µg/kg and 125,0 µg/kg) and three ochtatoxin A. In two samples evaluated amount of ochratoxin A exceeded the tolerated level.

Keywords: spelt, *Fusarium* toxigenic fungi, mycotoxins

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Ochratoxin A concentration in breast milk samples from Germany

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Ochratoxin A (OTA) is a well-known nephrotoxic mycotoxin and has been classified as potential human carcinogen. Based on toxicological information, maximum levels in certain foods have been set and tolerable daily intake (TDI) values have been proposed [1]. Ingested OTA is excreted with human milk and biomonitoring studies conducted in different countries have shown a wide range of OTA concentrations [Table 1 in 2]. Mother's milk is the best form of nutrition early in life, yet it may contain contaminants. The aim of this study was to assess infants' exposure to OTA with milk from two German areas (Dortmund and Hannover).
Breast milk samples (colostrum, transitory and mature milk) were obtained from 90 mothers who had signed an informed consent sheet. The previously validated analytical method (LOD: 10 ng/L) involves liquid-liquid extraction and analysis by HPLC with tandem mass spectrometric detection [2].
More than 50% of all milk samples contained detectable OTA levels. Overall, at three stages of nursing the average concentrations in milk of the Dortmund cohort were slightly higher than those measured in the Hannover cohort (Table). The highest observed concentration of 100 ng/L was found in a colostrum sample from Dortmund.

Table: OTA Levels (ng/L) in breast milk samples from Dortmund and Hannover cohort

Total milk samples	Colostrum ± SD	Transitory milk ± SD	Mature milk ± SD
Dortmund (30 samples)	26.2 ± 20.1	17.9 ± 10.4	22.6 ± 22.0
Hannover (60 samples)	12.4 ± 17.1	14.1 ± 16.7	10.5 ± 17.5

The exposure of infants was assessed by calculating their OTA-intake with human milk. In general, the TDI of 5 ng/kg-bw/day, set for adults, was not exceeded. Only with 5 milk samples, TDI values of 11 ng/kg-bw/day were calculated. To assess also the OTA exposure of infants from infant formulae, 6 commercial products from the German local market were analyzed by IAC and LC-MS/MS. Only 1 product was tested positive for OTA (17.7 ng/L).
In conclusion, infant exposure to OTA with human milk and/or infant formulae in Germany is lower compared to some other countries. Yet, preliminary data indicate also the presence of deoxynivalenol in human milk. Thus, simultaneous exposure of infants to OTA and other mycotoxins should be further investigated.

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Mycotoxin contamination levels in European feed samples 2010

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In the year 2010, 1 166 samples from Europe (mainly from Central Europe) were analyzed for the most important mycotoxins: aflatoxins (Afla), zearalenone (ZON), B-trichothecenes - deoxynivalenol and acetyldeoxynivalenol (DON), fumonisins (FUM) and ochratoxin A (OTA). Analyses were performed either by HPLC (high performance liquid chromatography) or ELISA (enzyme-linked immunosorbent assay) according to standard procedures. Raw materials and grains were preferably analysed by ELISA, whereas for compound feed and feed premixes HPLC was used.

Out of the more than 1 100 samples analyzed within this 12-months period, 36% were negative for the presence of mycotoxins (below the limits of detection). 28%, 23%, 59%, 50% and 28% of all samples tested positive for contamination with Afla, ZON, DON, FUM and OTA, respectively. The *Fusarium* mycotoxins DON was the most frequently occurring mycotoxin in Europe followed by FUM. If compared with data from the previous year percentage of positive ranged within the same area for all tested mycotoxins. On the other side, average contamination levels of all samples analyzed were higher for certain mycotoxins. Especially in the case of DON the values increased from 404 µg/kg in the year 2009 to 907 µg/kg (median of samples tested positive: 490 µg/kg) in 2010 (median of samples tested positive: 561 µg/kg). The maximum DON-contamination value found was as high as 49 000 µg/kg in a wheat sample sourced in Austria. About 50% of all the surveyed samples showed a DON contamination above 300 µg/kg and 20% of all the feed samples were above the EU guidance values for DON (900 µg/kg) in complementary and complete feedingstuffs for pigs (EC, 2006). In the case of FUM, 9% of the analysed samples were higher than the respective guideline levels (complementary and complete feedingstuffs) for pigs.

Mycotoxin contamination in animal diets represents an ubiquitous problem for animal performance and health; thus a reliable mycotoxin risk management is a prerequisite.

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„MYCOMON“ (1999-2009) – monitoring of afla- and ochra-toxigenic microfungi in foods

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Introduction: Toxigenic microfungi are microorganisms capable of producing mycotoxins. They are important factors that may have a potentially negative effect on human health. The most important toxigenic microfungi are the producers of aflatoxins (AFs) and ochratoxin A (OTA). Foodstuffs are suitable substrates for the contamination, growth and propagation of toxigenic microfungi and for the production of mycotoxins. A study „MYCOMON” of the occurrence of toxigenic microfungi, producers of AFs and OTA, in selected commodities of a consumer food basket was prepared. Investigations in this study were incorporated into the National Environment and Health Monitoring Programme in the Czech Republic. Its objective was to obtain information on the degree of the immediate contamination of foodstuffs with toxigenic microfungi which could serve as the basis for the evaluation of the dietary exposure and health risk assessment of afla- and ochra- toxigenic microfungi in the Czech foods.

Materials and Methods: Twenty-one commodities were purchased from retail at 12 places in 4 terms (March, May, September, November) during the years 1999–2009. It presents 2016 foodstuffs samples in all. Aflatoxigenic microfungi *Aspergillus* Section *Flavi* (e.g. *Aspergillus flavus*, *A. parasiticus*) and ochratoxigenic microfungi (*Aspergillus ochraceus*, *A.* Section *Nigri*, *Penicillium verrucosum*, *P. nordicum*) were monitored in foods. Classical mycological methods were used. Chromogenic media (AFPA medium, ADMB medium, DRYES agar and PRYES agar) were used for identification of toxigenic microfungi. HPTLC determination of AFs in mycelium of microfungi on YES medium was carried out.

Results and Conclusions: Frequency data of the qualitative and quantitative incidence of toxigenic microfungi for these commodities were obtained. 140 (46% toxigenic) strains of *Aspergillus flavus* were isolated from foodstuffs (black tea, fruit tea, caraway seeds, fine semolina, fine flour, granular flour, semi-granular flour, sweet red pepper, black pepper, raisins, rice, pasta, oat flakes, semolina, walnuts, lentils, and peas), 303 strains of *Aspergillus* Section *Nigri* were isolated from foodstuffs (black tea, fruit tea, caraway seeds, sweet red pepper, black pepper, raisins, rice, fine flour, granular flour, fine semolina and oat flakes). *Aspergillus parasiticus*, *A. Nomius, A. ochraceus*, *Penicillium verrucosum*, *P. nordicum* were not isolated from the tested foods. The toxigenic microfungi are stored in a solution of 30% glycerol in deep-freezer box by -71°C for further research by molecular biological methods.

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

The determination of *Fusarium* mycotoxins in grains from eleven events of transgenic Bt-maize

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Introduction: Maize (*Zea mays* L.) is cultivated on large areas world-wide and serves as a staple food for millions of inhabitants. Maize is, however, a particularly problematic commodity in the context of aflatoxins and *Fusarium* mycotoxins (Strosnider et al 2006). Bt maize is primary an important potential tool for insect pest protection, both in the EU and in other countries. Effective measures to combat pests therefore often have a positive side-effect in that they also reduce *Fusarium* mycotoxin levels (Wu 2007, Ostry et al. 2010). The aim of this preliminary study was a determination of *Fusarium* mycotoxins in grains from transgenic Bt-maize.
Materials and Methods: The grains from eleven events of transgenic Bt-maize (MON810; NK603xMON810; 1507x59122xNK603; MON88017; MIR604; MIR162; Bt11x GA21; Bt11xMIR604xGA21; Bt11xMIR162xMIR604xGA21; MON89034xNK603; MON89034xMON88017) were collected in 2002-2009 within project with Czech Ministry of Environment. The HPLC-FD and LC-MS/MS methods were employed for purposes of the twelve *Fusarium* mycotoxins (FUMB1, FUMB2, DON, 3-AcDON, 15-AcDON, DON-3Glu, NIV, T-2 toxin, HT-2 toxin, ZON, DAS, FUS X) quantification.
Results and Conclusions: FUMB1 was detected in low concentration (26.6-298.0 ng/g) in 9 samples, FUMB2 was detected in low concentration (34.3-85.9 ng/g) in 4 from 11 samples of events of transgenic Bt-maize. DON was detected in very low concentration (2.4-4.7 ng/g) in 3 samples and DON-3Glu was detected in low concentration (3.2-7.9 ng/g) in 8 from 11 samples of events of transgenic Bt-maize. The results of further *Fusarium* mycotoxins were under the limit of quantification of methods.

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Preliminary exposure assessment of fumonisin B1 and B2 in the Czech Republic

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Introduction: Fumonisin B1 (FUM B1) and fumonisin B2 (FUM B2) are important mycotoxins produced by several *Fusarium* spp., including *Fusarium verticillioides* and *Fusarium proliferatum*. They occur worldwide and are found predominantly in maize and maize products, but more recently they have also been found in other food commodities, such as rice, sorghum, asparagus and mung beans. Toxicity testing in animals shows that the liver and kidneys are the main targets for FUMs toxicity, especially in rodents. Cardiovascular effects have also been reported. The basis for the toxicity of fumonisins is thought to be interference with the synthesis of complex glycol-sphingolipids, which has effects on cell growth, development and function. Very high oesophageal cancer incidences observed in human populations in the Transkei of South Africa were correlated with high intake of maize as staple food and the high concentration of FUMs, particularly FUMB1 in this food. The International Agency for Research on Cancer characterized the fumonisins as Group 2B carcinogens. The JECFA allocated a group provisional maximum tolerable daily intake (PMTDI) of 2000 ng/kg of body weight to FUM B1, B2, and B3, alone or in combination, on the basis of the NOEL of 0.2 mg/kg body weight per day and a safety factor of 100. Scientific Committee on Food determined comparable tolerable daily intake (**TDI**) **2000 ng/kg b.w./day** to FUM B1, B2, and B3, alone or in combination. However, at present the available data on exposure via maize foodstuffs are incomplete in the Czech Republic. The estimation of dietary exposure dose of FUMs for the Czech population (for average person of 64 kg b.w.) was for maize products **21 ng/kg b.w./day** in the 90th years (Ostry and Ruprich 1998).

Materials and Methods: Total **48 samples** of maize flour were collected at 12 places in four terms in Czech retail **in year 2010**. Samples of maize flour were purified on commercial immunoaffinity columns (FUMONIPREP®, R-Biopharm AG). The validated HPLC-FD method was used for quantification of FUM B1 and FUM B2 in maize flour. The limit of quantification (**LOQ**) of the method for FUM B1 and FUM B2 was **25 ng/g**. Average dietary exposure will be calculated by multiplying of FUM B1 and FUM B2 concentration data for samples of maize products with consumption rate per kg of b.w. per day.

Results and Conclusions: The estimation of dietary exposure dose of FUM B1 and B2 from **maize flour** for the Czech population and patients with coeliac disease was **3 ng/kg b.w./day** and **13 ng/kg b.w./day** respectively. This estimate can then be compared to known **TDI** to assess if dietary exposure represents a risk to consumer health.

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Analytical aspects of the monitoring of the occurrence of T2 and HT2 in Dutch food by dublicate diet collection in the peroid 1976-2006

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Duplicate diet studies are an indispensable part of toxicological evaluation of contaminants in food. As 24-hour duplicate diet compilation results in highly diluted samples it is of utmost importance to have analytical methods available which combine reliability and ruggedness with extremely low limits of quantitation (LOQ).
For the purpose of the analysis of T2 and HT2 toxin in duplicate diets a GC-MS method was developed and validated. For T2 and HT2 toxin an LOQ of 0,010 µg/kg was obtained with a relative standard deviation (sextuplicate analysis, repeatability conditions, n=8) ranging of 2.2-5.9% for HT2 (0.061-0.222 µg/kg) and 4.4-24.2% for T2 (0.011-0.061 µg/kg). The reproducibility ranged from 1.0-4.1% for HT2, respectively 0.3-11.3% for T2 and was calculated by duplicate analysis of seven individual samples on two different measurement sequences. The recovery of HT2 ranged from 93.6-101.6% and of T2 from 90.6-106.3% (n=6).
As of 2001 a combined temporary tolerable daily intake (tTDI) for T2 and HT2 toxins of 0.061 g/kg body weight/day exist, established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA). By analysing duplicate diets dating from 1976, 1978, 1984 (spring and autumn), 1994 (spring and autumn) and 2004 (spring and autumn) an accurate dietary intake calculation of human exposure to T2 and HT2 was possible for adults. During spring and autumn of 2006 duplicate diets were collected of infants (2-6 years of age). Examples of the dietary intake of T2 and HT2 toxin of adults and infants will be presented.

Worldwide contamination levels of feedstuffs and feed

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The occurrence of mycotoxins is known to be ubiquitous and related to many deleterious effects on humans and animals. Several scientific papers focus on the effects of one or combinations of mycotoxins in animals, usually ascertained by trials carried out with artificially contaminated diets. Studies on the actual contamination likely to be found in practical feedstuffs and feed are not common as the majority of surveys analyse food intended for human consumption, or focus on a certain country or region, on a certain commodity, and usually the amount of samples tested is too small to allow generalizations. This work on the 12-months period between January 2010 and December 2010 during which a total of 3 349 samples were analyzed and in total 6 488 analyses were carried out for the most important mycotoxins in terms of agriculture and animal production – aflatoxins (Afla), zearalenone (ZON), deoxynivalenol (DON), fumonisins (FUM) and ochratoxin A (OTA). 70% of the samples were analyzed by HPLC, 30% by ELISA and aprox. 9% of the samples were submitted to a TLC method. For the purpose of data analysis, non-detection levels were based on the quantification limits of the test method for each mycotoxin. Interesting results were obtained in this survey regarding the worldwide distribution of mycotoxins and distribution of contamination within the various contamination levels (Figure 1).

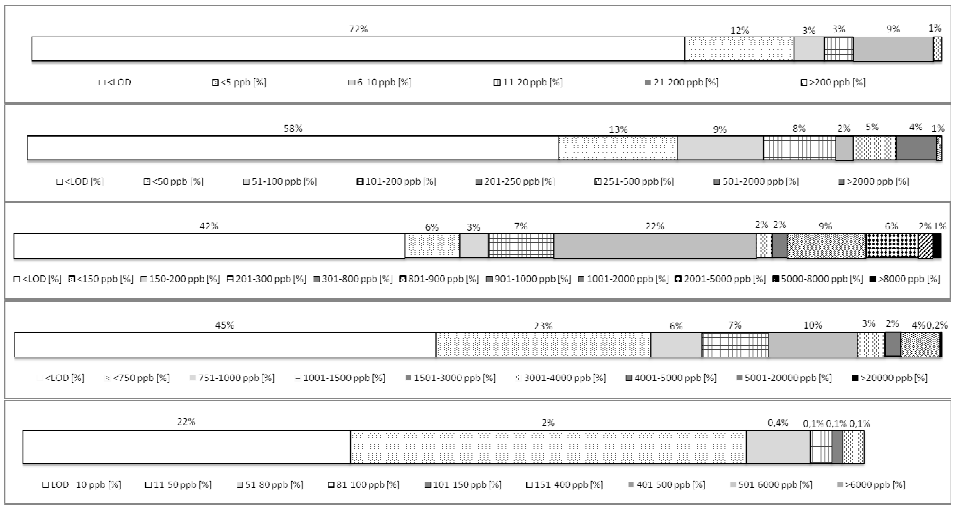

Figure 1: Distribution of Afla, ZON, DON, FUM and OTA (from up to bottom) contamination results by contamination levels

10% of tested samples presented contamination levels higher than 20 ppb Afla, the highest permitted level of aflatoxin B1 in all feed materials (2003/100/EC). As for ZON, 30% of samples were contaminated with 50 ppb and higher. Interestingly, the majority of DON contaminated samples were inserted in the contamination range from 301 to 800 ppb DON. Approximately 20% of FUM contaminated samples presented levels of 1500 ppb and higher. The majority (74%) of samples tested below the LOD (limit of detection) for OTA.

Reference

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German rye harvest 2010: Occurrence, distribution and correlation of ergot alkaloids and sclerotia

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Every year the Max Rubner-Institut (MRI) -Federal Research Institute of Nutrition and Food- analyses statistically taken samples from 12 (13) different Federal States in Germany according to the specific harvest and quality assessment (BEE). Samples were taken during harvest in the field and directly sent to MRI.
Three rye samples were pooled after grinding (500 µm) and screened for ergot alkaloids with the LC-MS/MS-methoda with a detection limit of 1 µg/kg. Positively screened pools were separated and further analysed individuallyb.
In 180 positively screened rye samples -out of 237- ergot alkaloids were determined between 3 and 1,498 µg/kg and sclerotia content ranged between 0 and 0.45%. The sclerotia content did not correlate well with the overall alkaloid content, which was lower than 100 µg/kg in 82.2% of the analysed rye samples (Tab.1). This indicates a low level of ergot alkaloid contamination in German rye in the year 2010.

Tab.1: Percentage proportion of alkaloid content in 180 German rye samples, harvest 2010

Content µg/kg	ergocornine/ ergocorninine	ergocristine/ ergocristinine	ergocryptine/ ergocryptinine	ergometrine/ ergometrinine	ergosine/ ergosinine	ergotamine/ ergotaminine	sum
>1300	-	-	-	-	-	-	0.6
>600	-	-	-	-	0.6	-	1.1
>300 - 600	-	-	-	-	-	1.1	4.4
>200 - 300	1.1	1.7	-	-	-	-	4.4
>100 - 200	1.1	5.6	1.1	-	2.2	4.4	7.2
>10 - 100	15.6	16.7	13.3	15.6	12.8	19.4	40
< 10	32.8	31.7	30.6	83.9	26.1	57.2	42.2
n.d.	49.4	44.4	55	0.6	58.3	17.8	0

n.d.: not detected

The six major ergot alkaloids and their isomers were distributed unevenly. Whereas in the southern parts of Germany ergotcristine/-inine and ergotamine/-inine seemed to be the detected alkaloids with the highest amounts in rye, the north-eastern parts showed more ergotcornine/-inine and ergotcryptine/-inine content in rye sclerotia. Also ergosine/-inine were analysed at some high amounts in different rye samples of Germany. However, in nearly all samples (99.4%) ergometrine/-inine could be detected at low levels (may be due to the lowest detection limit), making ergometrine/-inine an indicator for alkaloid content.

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The occurrence of zearalenone in milk and dairy products available in Czech market

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A survey of *Fusarium* mycotoxins deoxynivalenol (DON) and zearalenone (ZEA) was carried out in 51 samples of milk and dairy product available in Czech market. 34 samples originated in Czech Republic, the rest was from other EU countries (figure 1). The set of sample consist of pasteurised milk and dairy products with higher fat content and higher dry matter content (figure 2).
Samples were analysed using ELISA RidaScreen ZEA (limit of detection (LOD) = 18 ng.kg-1 and limit of quantification (LOQ) = 30 ng.kg-1) and ELISA RidaScreen DON (LOD = 1,8 µg.kg-1 a LOQ = 3,7 µg.kg-1).

Fig. 1: The origin of surveyed products

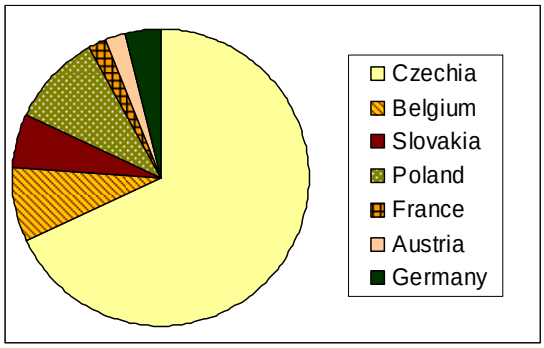
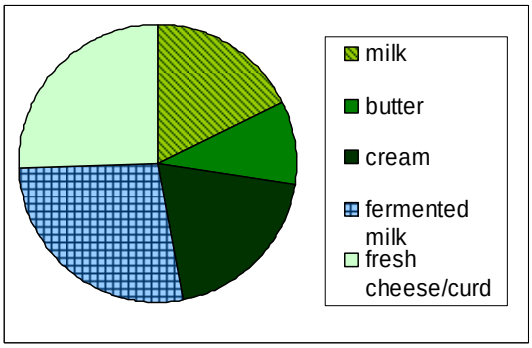


Fig 2: Milk products present in the surveyed sample set and their share



DON was under the LOD in all of the analysed samples. Zearalenone was found in 19 samples in the range 31 - 342 ng.kg-1. The concentration was comparable to those already found in other EU countries [1]. Eight curds/fresh cheeses (i. e. 62% of the group) contained zearalenon, as well as four fermented milks (29%), four samples of creams (40%), one sample of pasteurised milk (11%) and one butter sample (20%). This survey suggest that zearalenon is not cumulated in milk fat more than in dry matter, since the highest occurence of positive sample was in the group of curds and fresh cheeses. Although more than one third of samples contained ZEA, the concentrations are very low and the excess of tolerable daily intake 200 ng.kg-1 B.W. (according to ES 865/2005) should not threaten an average consumer of dairy products.

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The occurrence of mycotoxins in feed in Croatia

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Mycotoxins, the secondary metabolites of various fungi, are common contaminants of grains and animal feed. It is well known that consumption of mycotoxins, even in small quantities, can result in intoxication. Therefore, worldwide occurrence and a habit to use less contaminated grains for production of animal feed forced the researchers to identify "safe levels" of these toxins in grains and feed. As basis for prevention of intoxication of animals with mycotoxins and for risk analysis it is necessary to continually monitor the occurrence of mycotoxins in grains and in feed. Many countries have established the survey programs of occurrence of mycotoxins in different matrices. In Croatia, there is still no monitoring program of mycotoxins in animal feed, and even though maximal limits of certain mycotoxins has been set, there is no adequate quantity of testing of ingredients and finished feed. Most of the tests are still done occasionally or in a case of problems in health of animals.
There are several methods for the analysis of mycotoxins including chromatographic methods like thin layer chromatography, high performance liquid chromatography, gas chromatography and different liquid chromatographic methods coupled with mass spectrometric detectors. In addition, enzyme-linked immunosorbent assays and similar assays enable quick and reliable screening of samples. In spite of improved methodology in mycotoxin testing that enables reaching adequate limits of detection and quantification for current legislative limits, the most limiting factor in selection of a suitable method is the cost of analysis. In Croatia, as well as in many other countries in the world, laboratories are still limited in instrumentation and available funds. Therefore, the most of the testing for the presence of mycotoxins is made with enzyme-linked immunosorbent assay and positive samples are confirmed and re-calculated with high-performance liquid chromatographic or thin-layer chromatographic method.
The aim of this paper is to provide a quick overview of the occurrence of selected mycotoxins in grains and feed in Croatia in a two year period. Additionally, most of the samples were also tested microbiologically for the presence of potential pathogenic moulds. Analysis of grains and feed samples for the presence of trichothecene mycotoxins, aflatoxin, zearalenone and ochratoxin showed contamination of feed or grains in 45.1% and 42.5% in 2009 and 2010 year, respectively. The feed was mostly contaminated with deoxynivalenol in quantities below 0.5 mg/kg. Mycotoxin deoxynivalenol was detected in 90.9% of contaminated feed samples in 2009 and in 69.7% of samples in 2010. Amont that, only 18.2% of tested samples in 2009 and 21.2% of samples in 2010 were contaminated with deoxynivalenol in quantities above 0.5 mg/kg of feed. Contamination of samples with T-2 toxin and diacetoxyscirpenol and fumonisin was detected in significantly smaller number of samples. Incidence of zearalenone and aflatoxin was significantly higher in 2010 (60.0% and 85.7%, respectively) in comparison to the contaminations of samples in 2009 (21.2% and 40.0%, respectively). At the same time, mycological analysis of grains and feed samples showed presence of fungi in 17.1% and 38.2% in 2009 and 2010 year, respectively. Occurrence of tested mycotoxins in different feeds and grains, as well as correlation with potentially pathogenic fungi is presented in this paper.

Presence of mycotoxins in corn-dried distiller's grains with soluble (corn-DDGS) in Thailand

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Dried distiller's grains with soluble (DDGS) is a by-product of the distillery process, produced commonly during the generation of corn-ethanol fuel. Usage of DDGS to replace corn as animal feed is of wide interest. Mycotoxin contamination in DDGS may harm animal health (Wu and Munkvold, 2008). However, little data of the co-occurrence of mycotoxins in DDGS, particularly at the end user (farmer) level, has been reported. The aim of this study was conducted to simultaneously detect six mycotoxins, including deoxyninalenol (DON), zealarenone (ZON), T-2 toxin (T-2), fumonisin B1 (FB1), fumonisin B2 (FB2) and beauvericin, in corn-DDGS used as animal feed by LC-MS/MS.

A total of 59 corn-DDGS samples collected from swine farms in central region of Thailand in 2009-2010 were tested. As depicted in Table 1, results showed the presence of DON, ZON, T-2, FB1, FB2 and beauvericin at various levels. Additionally, analysis of 50.8% of samples found the co-occurrence of five mycotoxins. No T-2 toxin was detected. Fumonisin was the predominant toxin detected in corn-DDGS. Interestingly, beauvericin could be detected in all fumonisin-contaminated-samples.

Table 1: Concentration of mycotoxins and number of detected corn-DDGS

Concentration (mg/kg)	DON	ZON	T-2	FB1	FB2	Beauvericin
ND	25	11	59	1	1	1
0.01-1.00	27	35	-	6	17	54
1.01-10.00	7	12	-	42	35	4
10.01-100	-	1	-	9	5	-
>100	-	-	-	1	1	-

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Wu, F. and Munkvold, G.P., 2008. Mycotoxins in ethanol co-products: modeling economic impacts on the livestock industry and management strategies. J Agric Food Chem., 56(11), 3900-11.

Evaluation of antifungal effect of extracts of pulp and leaves of *Argania spinosa* of Tindouf on an aflatoxin-producing strain of *Aspergillus flavus*

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Toxic mushrooms are a real danger because they contaminate food, these fungi secrete mycotoxins are secondary metabolites causing problems along the food chain may generate health risks. The use of medicinal plants with antimicrobial properties is a strong alternative interesting. Among the plant resources that have antimicrobial properties of *Argania spinosa*, a tree endemic of southwestern Algeria, in particular the region of Tindouf (Tindouf Nature Reserve).
The objective of this work is part of assessing the antifungal effect of extracts (aqueous and ethanolic macerate) derived from the leaves and pulp on *Aspergillus flavus* that produces aflatoxin. Assessing the antifungal activity of these extracts on solid medium and liquid PDAac and PDB respectively, reveals that they have exercised a significant inhibitory activity vis-à-vis the strain tested.
The ethanol extract of leaves showed strong antifungal activity with a percentage of the most significant inhibition and minimum inhibitory concentration of 125 mu.l on solid medium. The ethanol extract of the pulp induced a marked inhibition vis-à-vis the strain *A. flavus*. Ethanolic maceration of the pulp induced total inhibition of mycelial growth of *A. flavus* at a concentration 250 mu.l / ml at the 3, 4 and 5 days, the results also show that the MIC of the extract was collected at 125 mu.l / ml. The antifungal index was more significant for extracts and ethanolic maceration. It should be noted that the values of these indices were 76%, 63% and 74% respectively for ethanol extracts and macerate the leaves and pulp.

The statement focused on the results of the liquid medium is that each increase in the concentration of extracts in the culture medium it 'is a reduction in the amount of fungal biomass.
Moreover, the macerate leaves significantly inhibited the development of biomass, the latter was completely inhibited at the concentration 100 mu.l / ml for 14 days.

Keywords: *Aspergillus flavus* - *Argania Spinosa*-Tindouf- extracts of leaves and pulp-antifungal activity

Evaluation of antifungal activity of some oil (oil coloquinte – lin seed oil - black seed oil) on an aflatoxin-producing strain of *Aspergillus flavus*

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The aim of this study was undertaken in order to examine the antifungal activity of various concentrations of tree seeds oils: *Citrullus colocynthis* seeds oil (Coloquinte oil), *Linum usitatissimum* seeds oil (Linseed oil) and *Nigella sativa* seeds oil (Black Seed Oil), against Aflatoxigenic strains of *Aspergillus flavus*.
The lipid fraction (oil) was extracted using a Soxhlet apparatus; the oil content of the seeds ranged from **17.63** to **39.96%.**
The study of physicochemical characters attested that the indices values measured for our oils approach to those fixed by the Standard. This values were (**1.59 to 52.90 Mg KOH/g**), (**0.80 to 26.85%**) and (**5.68 to 30.01 Meq of O_2/kg**) respectively for the acid value, acidity and peroxide value.
The oils subject of this study showed a variable antifungal activity against *Aspergillus flavus* strains tested.
The evaluation of the radial growth on solid medium (**PDA**) reveals a corresponding decrease in fungal mycelial growth with increased concentration of oils added to the medium. The investigation of the antifungal indices, allows to classify our oils in the decreasing order as follows: linseed oil (**29%**) > coloquinte oil (**26.5%**) > black seed oil (**18.75%**).
Furthermore, mycelial dry weight was used to evaluate the growth of *A. flavus* strain on liquid medium (**PDB**), which revealed that Coloquinte oil and Linseed oil had a variable reduction. However, this reduction was noted when the concentrations added to the medium was higher or equal **80 µl/ml**. In the other hand the Black seed oil reduced the mycelial weight of *A. flavus* at the concentrations **80 µl/ml** and **100 µl/ml.**

Keywords: Antifungal activity, oils, seeds, *Citrullus colocynthis*, *Linum usitatissimum*, *Nigella sativa*, *Aspergillus flavus*

The role of dietary Mycofix® in reduction of DNA damage induced by deoxynivalenol in chicken blood lymphocytes

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Many compounds and treatments have been tested in order to reduce mycotoxin concentrations in food and feed or to alleviate their adverse effects on animals. Some of these treatments show promising prospects for commercial application, while others have had commercial applications already. However, there is a little information available regarding the effect of DON on DNA fragmentation in blood lymphocytes. In addition, the effects of the Mycofix® Plus (Biomin GTI GmbH, Herzogenburg, Austria) supplementation to deoxynivalenol contaminated broiler diets (low protein diets) on lymphocytes DNA have not yet been demonstrated. Therefore, the aim of the present study was to evaluate the effects of Mycofix® Plus supplementation on the lymphocyte DNA damage induced by DON in chickens.

Methods: Thirty two 1-d-old broiler chicks were randomly divided into four groups. The control group was fed non-contaminated diet (diet with low protein) and a second group supplemented with Mycofix® Plus (Biomin GmbH, Herzogenburg, Austria). Another group of broilers was fed a diet artificially contaminated with 10 mg DON/kg diet, whereas another group was fed the DON-contaminated diets supplemented with Mycofix® Plus. The normal starter and grower diets (low protein) were provided ad libitum for 2 and 3 weeks. Chicks were weighed individually, and feed consumption for each pen was measured weekly during the 5-wk experiment. Birds were vaccinated at the hatchery with one dose per chick of Mild-Vac-M vaccine to Infectious Bronchitis (IB) by coarse aerosol spray at the first day of life. On day 35 at the end of experiment, blood was collected from 8 chicks per group during slaughtering, and the degree of lymphocyte DNA damage was measured in plasma by Comet assay.

Results: DON significantly increased the amount of DNA damage (40.37%) in chicken lymphocytes presented as the portion of DNA in the tail of comet compared with the control group (27.74%). However, Mycofix® Plus supplementation significantly reduced the percentage of DNA damage in the tail in DON with Mycofix® Plus treated low protein fed group (24.72%).

Conclusion: The rapidly proliferating cells of the immune system are primarily affected by this mycotoxin due to the principal mode of action of trichothecene mycotoxin, which includes inhibition of protein synthesis and/or protein deficiency. Our results demonstrated that the diets contaminated with the mycotoxin DON at moderate levels are already able to induce lymphocyte DNA damage in chickens. Supplementation with Mycofix® Plus protected lymphocyte DNA from toxin impact and it can be beneficial for remaining the lymphocyte DNA integrity.

On the immunotoxic effects of Mycofix® on broilers when fed deoxynivalenol contaminated low protein grower diets

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One of the major concerns for the poultry industry is the threat of mycotoxins associated with poultry feedstuffs. It has been emphasized that a certain risk remains of mycotoxin production by several *Fusarium* species despite application of a number of preventive steps in plant production, because of the high influence of the weather conditions at the time of cereal flowering. The aim of the present study was to evaluate the effects of Mycofix® Plus dietary supplementation in either the presence or the absence of DON concerning the immunosuppressing IB-titer effects.

Methods: Thirty two 1-d-old broiler chicks were randomly divided into four groups. The control group was fed non-contaminated diet (starter diet containing normal protein, 21%) and a second group supplemented with Mycofix® Plus (Biomin GmbH, Herzogenburg, Austria). The third group of broilers was fed a diet artificially contaminated with 10 mg DON/kg diet, whereas the fourth group was fed the DON-contaminated diets supplemented with Mycofix® Plus. The starter and low protein grower diets were provided *ad libitum* for 2 and 3 weeks. Chicks were weighed individually, and feed consumption for each pen was measured weekly during the 5-wk experiment. Birds were vaccinated at the hatchery with one dose per chick of Mild-Vac-M vaccine to Infectious viral Bronchitis (IBV) by coarse aerosol spray at the first day of life. On day 35 at the end of experiment, blood was collected from 8 chicks per group during slaughtering, and the serum was separated. The antibody titres for IBV were measured by the anti-IB antibody ELISA kit.

Results: Feeding of the *Fusarium* toxin contaminated diets to broilers resulted in a significant decrease in performance as indicated by a depression in feed intake and live body weight (BW) gain. Mycofix® Plus addition to the contaminated diets resulted in a significantly improved broiler performance, indicating that the addition of Mycofix Plus as a feed additive to the DON contaminated feed of the broilers effectively alleviated the alterations caused by DON and led to comparable body weight and BW gain as in the control group. In the present experiment, we found also that the feeding of DON contaminated diets decreased ($P < 0.05$) serum antibody titre against IBV (802 IU). However, it was found that the antibody titres to the IB to be significantly improved when DON contaminated diets were supplemented with Mycofix (1155 IU). Moreover, there were no significant difference between Mycofix supplemented group (1599 IU) and control group (1570 IU).

Conclusion: It can be concluded from the present results that DON negatively affected performance of broilers and depressed serum titres to IB. However, the dietary Mycofix® Plus supplementation as a detoxifying agent was successful in overcoming the protein deficient and/or mycotoxin-related effects.

Effect of different silicates clay minerals on decreasing the mycotoxins in food stuff and animal feeds

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The contamination of animal feed and human foodstuff by highly poisonous low molecular weight natural products of secondary metabolites produced by filamentous fungi, especially the species of *Aspergillus*, *Fusarium, Penicillium*, *Claviceps* and *Alternarian* called Mycotoxins represent a worldwide problem to farmers and food industries. Mycotoxins-containing feed can cause serious diseases to farm animals resulting to suffering, death and big economic losses. This thesis research focuses on the analysis of the effect of different silicates clay minerals (SCM) on decreasing the bioavailability of common Mycotoxins like Aflatoxin M1 and Ochratoxin A. These SCMs contain exchangeable cations which are effective adsorbents, and can efficiently bind the mycotoxins in the gastro-intestinal tract of animals and reduced their adversity. In this study the natural SCMs will be assayed *in vitro* at conditions similar to the gastro-intestinal tract of animals on different mycotoxins. Measurement of the residual Mycotoxins will be performed by high performance liquid chromatography HPLC coupled with a diode array UV detector (UV/DAD) or fluorescence detector (FD) in order to determine the binding capacities of the clays.

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Zearalenone adsorption by organoclays – correlation of *in vitro* with *in vivo* findings

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Zearalenone (ZON) is an important mycotoxin in warm and temperate regions. It is produced by several members of the genus *Fusarium* and shows estrogenic effects on mammalian reproductive systems. One strategy to counteract ZON taken in with contaminated feedstuff is the addition of non-nutritive adsorbent materials which sequester the ZON before resorption in the gastro-intestinal tract of the animal.
The aim of this study was to test various adsorbents *in vitro* and subsequently correlate these results with data obtained in *in vivo* studies with gilts. The adsorbents used in these tests were 2 organoclays (S1, S2), one humic acid based product (S3) and Cholestyramine (S4). The latter has proven its efficacy in several *in vitro* tests in literature.
The performed *in vitro* tests included adsorption tests in buffer solutions as well as adsorption isotherms.
For the *in vivo* tests a completely randomized design was used with five 5-week-old gilts assigned to each dietary treatment beginning on day 14 post weaning. Dietary treatments evaluated included a negative control (no ZON), a positive control (ZON) and treatment groups receiving ZON and 0.5% of one of the 4 adsorbents. The data presented here were obtained in 2 individual feeding trials carried out at different ZON concentrations. The materials S1 and S2 were tested at a concentration of 1.0 µg/kg ZON, the second test (S3 and S4) was carried out at 1.5 µg/kg ZON.
This study showed that despite of very promising *in vitro* test results none of the tested materials was able to prevent or reduce the effects of ZON at the tested concentrations in weanling pigs.

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Adjunction of yeast cell walls or live yeast decrease ochratoxin A genotoxicity in poultry

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Ochratoxin A (OTA) is a mycotoxin contaminating mainly cereals but also other crops such bean, raisin, cocoa, coffee. OTA can be accumulated in meat, and is not destroyed by food processing. It is responsible of nephrotoxicity in pig and poultry, and is implicated in Balkan endemic nephropathy and associated urothelial cancer in human [1]. OTA is genotoxic and forms covalent DNA adduct, notably C-C8 dG OTA after biotransformation [2,3,4]. One of the most promising and economical strategy for reducing the animal exposure to mycotoxins is the utilization of adsorbents in animal feed to reduce gastrointestinal absorption of mycotoxins. Besides its excellent nutritional value, yeast or yeast cell walls show a potential as mycotoxin binders. The aim of this paper is to evaluate the capacity of several yeast cell walls and live yeast to decrease genotoxicity of OTA and establish if the decrease is only due to adsorption of OTA on yeast product. For this purpose, first we analyse the binding capacity of different yeast products, *in vitro*. Second, 10 poultries per group were fed two days with feed including yeast product, and then were fed seven days with feed including yeast product and OTA. DNA-adduct and OTA derivatives were analysed in liver and kidney of poultry. Depending of the amino acids, carbohydrates and lipids compositions of the yeast cell walls DNA adduct (reflecting genotoxicity) decrease or increase. The decrease observed with life yeast was not only correlated to adsorption but also to biotransformation of OTA which is modulated by yeast. Interestingly, DNA adduct patterns were correlated with OTA derivatives forms in the kidney. Although the adsorption capacity of activated carbon is nearly 100%, the detoxification is not complete.

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Characterization of the aminotransferase FumI from *Sphingopyxis sp.* MTA144 for deamination of hydrolyzed fumonisin B1

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The alpha-proteobacterium *Sphingopyxis sp*. MTA144 can degrade fumonisin B1 and related fumonisins, and a key enzyme in the degradation pathway is the aminotransferase FumI which deaminates the first intermediate in the catabolic pathway, the hydrolyzed fumonisin B1. We used a preparation of purified, His-tagged FumI, produced recombinantly in *Escherichia coli* in soluble form, for enzyme characterization to evaluate its potential as a feed enzyme for fumonisin detoxification in the gastrointestinal tract of animals.
The reaction product was identified by NMR as a 2-keto hydrolyzed fumonisin B1. Pyruvate was the preferred co-substrate and amino group acceptor. Addition of the co-enzyme pyridoxal phosphate enhanced the activity of FumI, and saturation was already reached at the lowest tested concentration of 10 µM. The enzyme was active over a broad temperature range between 6°C and 50°C with an optimum at 35°C and within the range of pH 6.5 – 9.7 with a rather sharp optimum from pH 8.0 to 8.5. It showed no activity at pH values up to 5 and activity could be recovered after preincubation for 1 h at pH 4.0 or higher, but not lower. Preincubation at various temperatures resulted in a steady decrease of enzyme activity with increasing temperature, and the aminotransferase was denaturated after preincubation at 60°C. The kinetic parameters K_M = 1.1 µM and k_{cat} = 104/min were determined with 5 mM pyruvate as co-substrate at optimal conditions. Specific activity was 2.2 µmol/min/mg.
Based on the enzyme characteristics, a technological application of the aminotransferase FumI seems possible, if the enzyme properties are considered.

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Efficiency of citric acid for the detoxification of aflatoxins in red chilli samples from Pakistan

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Aflatoxins (AFs) are toxic chemical compounds produced by various fungi that can contaminate different food and food products. AFs contamination was determined in 30 whole and 22 of ground red chili samples from Punjab, Pakistan, by HPLC equipped with fluorescence detector. The limits of detection and quantification were 0.05 and 0.15 µg/kg, respectively. Sixteen (53%) and 14 (64%) samples of whole and ground chilies, respectively, were found contaminated with AFs. The mean concentration in ground red chilies (23.2 µg/kg) was higher statistically than in whole red chilies (14.7 µg/kg). The results demonstrated that 84% decontamination was observed in ground red chilies compared to 74% in whole red chilies when the concentration of 1 N of citric acid was employed. The undertaken method of decontamination was easy to operate, rapid and effective not only in chilies but other food commodities as well.

Effect of fermentation parameters of rye dough on the content of deoxynivalenol and T2-toxin

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Dough fermentation is a process that reduces the toxicity of mycotoxins. Appropriately chosen method of fermentation and the type of micro-organisms taking part in it could significantly reduce the mycotoxin content [Lancova et al. 2008, Lesnik et al. 2008]. But what happens when the material is heavily contaminated with them? Are these methods so effective that the product obtained from such raw material could be safe?
The aim of this study was to determine the degree of decomposition of deoxynivalenol (DON) and T2-toxin from samples heavily contaminated by these mycotoxins using different methods of fermentation.
The culture of fungi of the genus *Fusarium* (*F.*) species, *F. culmorum*, *F. graminearum* was performed in a mineral medium, then rye grain with them was infected. Flour from grain heavily affected with fungi was prepared and samples were performed using direct fermentation of the dough with the addition of lactic acid (single phase) and three phase method (in sour soup). After the fermentation breadmaking was performed. The concentration of mycotoxin was marked with the use of the direct competence ELISA tests - AgraQuant Test Kit (Romer Labs).
After the milling of contaminated grains, the bran fraction had much more mycotoxins compared to flour. Content of DON and T2-toxin in the crust bread was slightly lower than in the crumb. The greatest reduction in both mycotoxins in the crumb was obtained in the direct method: T2-toxin from flour contaminated with *F.* species and DON from flour contaminated with *F. graminearum*. The content of DON in bread from less polluted rye after baking has decreased, while in the more polluted bread increased (above 1.5 mg/kg).
However, the mycotoxin content in all tested breads was higher than the Maximum Residue Limits (MRLs).

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Impact of wild-endophyte-infected perennial ryegrass pasture on ewe lambs and the efficacy of a mycotoxin deactivating product

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Fungal endophytes (*Neotyphodium lolii* and *N. coenophialum*) are known to infect perennial ryegrass (*Lolium perenne*) and tall fescue (*Lolium arundinaceum*) respectively. They produce alkaloids that impair animal health and performance (Perennial ryegrass and Fescue toxicoses). Animals grazing these pastures can experience reduced intake, heat stress and have low conception rates and lower survival of offspring.
An experiment was conducted in Victoria, Australia (Reed et al., 2011), to evaluate the effectiveness of a mycotoxin deactivator product (MDP) in ruminants exposed to endophyte toxins. Sixty multi-vaccinated Coopworth ewe lambs were selected for uniform age, live weight and condition at weaning. The sheep were given access to the experimental areas and introduced to a daily supplement of crushed barley (80%) and peas (20%) at 100 g/head. The 6 month-old lambs were allocated into two different groups of 30 animals each. One group received a supplement containing MDP (5%) and the other group received the same supplement but without MDP. The two groups rotated weekly between two paddocks so that each group grazed the same pasture for the duration of the trial. The pasture consisted of mature, low quality perennial ryegrass. The parameters evaluated in this trial included live weight and sheep behaviour (number of sheep seeking shade, grazing, at trough or huddled). During the trial the concentration of the endophyte-produced toxins, ergovaline and lolitrem B in the ryegrass both remained within low levels, viz. 0.5-1.0 mg/kg DM.
After 84 days the mean live weight was greater ($P<0.01$) for the treatment group (ADWG: 42 g/day) than for the control group (ADWG: 23 g/day). Low lamb growth rates were already expected because of the "mature low quality pasture" at that time of the year (hot dry summer).
Moreover, sheep without MDP exhibited slight signs of ill-thrift and heat stress but none exhibited staggers or any other signs suggestive of neurotoxicity. The main difference observed between the two flocks during this period was the greater use of shade by the MDP treated group ($P<0.001$), while the control group made little use of shade despite high temperatures (25-38°C) and a higher respiration rate ($P<0.01$). MDP is tentatively regarded as a management tool with some potential to improve the health of young ewes grazing wild endophyte-infected perennial ryegrass.

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Current mycotoxin research at the BAM Federal Institute for Materials Research and Testing

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This talk is intended to give a brief overview of the current, mycotoxin related research activities at the BAM Federal Institute of Materials Research and Testing. BAM's mission is to improve safety in technology and chemistry through research and development, testing, analysis, approvals, advice and information. In the case of food mycotoxins, the focus of interest lies on quantitative analysis and associated issues such as:

1) The production of matrix-matched certified reference materials (CRMs) for mycotoxins in various foods

BAM has recently produced two CRMs for ochratoxin A (in coffee: ERM®-BD475 and in wine: ERM®-BD476) as well as one CRM for deoxynivalenol (DON), nivalenol (NIV) and zearalenone (ZON) in wheat (ERM®-BC600). All CRMs were certified in the frame of the European Reference Material (ERM®) initiative. Ongoing projects concern as well as T-2/HT-2 toxins in oat flakes. Characteristics of the materials and the development process will be outlined.

2) The development of innovative analytical methods

BAM has recently developed methods for "problematic" mycotoxin/matrix combinations such as ZON occurring in edible oils (European maximum level for zearalenone in refined maize oil: 400 µg/kg) [1]. A novel approach based on dynamic covalent hydrazine chemistry was chosen to extract ZON from the oil. Method details and results of a recent interlaboratory comparison study "ZON in maize oil" conducted by BAM with the collaboration of ten analytical laboratories will be shown.

3) Studies on mycotoxin transformation processes

Mycotoxin transformation (degradation, conjugation etc.) in the field or during food processing can affect analytical results and, in some cases, lead to an underestimation of mycotoxin exposure. *In vivo*, chemical transformations can cause a detoxification or an increase in toxicity (e.g. in the case of the metabolic epoxidation of aflatoxin B1).
BAM has recently adopted an *in vitro* digestion model based on DIN 19738 to study the gastrointestinal degradation of DON, ZON and their known conjugates. Details on this approach and first results will be shown. Furthermore, new aspects of "masked" mycotoxin formation during food production and processing will be discussed.

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Deoxynivalenol detoxification capability of various bacterial isolates in rumen fluid

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Trichothecenes are a group of mycotoxins mainly produced by species of the fungus *Fusarium* while infecting plants on the field. Deoxynivalenol (DON) is one of the most abundant trichothecenes in food and feed. Due to its immunosupressive effects and impairment of the gastrointestinal system it presents a potential health risk to men and especially to farm animals, which mainly feed on cereal crops. Although preventive strategies have been implemented in agriculture, it is not always possible to avoid DON-contamination of crops and forages. Physical and chemical methods for the elimination of DON remained unsatisfactory. An effective alternative may be the use of microorganisms able to transform DON into non-toxic metabolites prior to the resorption in the intestinal tract as a feed additive.

The aim of this work was to test environmental bacterial isolates derived from rumen fluid, chicken intestine and soil for their DON-detoxification potential. Degradation experiments were performed in rumen fluid to simulate natural conditions of the intestinal tract of animals. Therefore DON (5 mg/L) was incubated with intact or sterilized rumen fluid in the presence or absence of bacterial isolates at 37°C. Liquid chromatography – mass spectrometry (HPLC-MS) was used to determine DON concentration after 5, 18, 26 and 48 hours of incubation.

Naturally occurring microbes in rumen fluid were able to detoxify DON. Autoclaved rumen fluid did not show any degradation ability. The addition of rumen and intestine bacterial isolates led to an increase of DON degradation rate in intact rumen fluid, whereas the soil-derived bacterial isolate had no effect on the natural detoxification capability of rumen fluid. The chicken isolate significantly reduced DON in sterilized rumen fluid too. DOM-1, the much less toxic deepoxide of DON, was identified as the sole metabolite of DON degradation.

The presence of rumen and chicken intestine isolates improved degradation ability of the natural rumen flora, which shows that these microbes are promising candidates for the development of a mycotoxin deactivating feed additive.

The fate of deoxynivaleol in wheat grits during extrusion cooking

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Deoxynivalenol (DON) is a toxic secondary metabolite produced by molds of the *Fusarium* genus and is known to cause a spectrum of diseases in animals. To study the effect of some additives on the reduction of DON during extrusion cooking, sodium bicarbonate (NaHCO3) and gluten were added to wheat grits spiked with DON (500 µg/kg). The samples were extruded under different pH values (6.0, 7.3, 7.8 and 8.0), protein contents (0, 8, 18.6, 26.6%), moisture contents (17 and 34%), and temperatures (100, 110, 120, 130, 140, 150, 160, 170, and 180°C). The reduction of DON during extrusion was quantified by stable isotope dilution analysis using 15-d1-DON as internal standard. Degradation products were monitored by HPLC-ESI-MS/MS. DON was relatively heat stable and the extrusion decreased DON by 3.1-37.8% for screw 1/1 and 17.8-26.5% for screw 3/1. The greatest reduction of DON was observed at 170 °C (37.8%) and 120 °C (26.5%) for screw 1/1 and 3/1, respectively. However, DON levels were dramatically reduced under alkaline conditions. A great reduction (79.2%) was observed in the grits with pH 8.0 at 150°C. Also a higher protein content in grits showed a greater reduction of DON. Moisture content seems to provide also a strong influence at relatively low temperatures (100-140°C). The radial expansion ratio was measured and found to be almost inversely proportional to the reduction. norDON B and nor DON C were identified as degradation products. This study provides crucial information for the risk assessment of extruded foods and feeds.

Keywords: deoxynivalenol; extrusion; reduction; degradation products; pH value; protein; moisture

The acute effects of deoxynivalenol on nutrient transporters trafficking in the gut epithelium of chickens

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The digestive tract is a target for the mycotoxin deoxynivalenol (DON). It is a major cereals grain contaminant of public health concern and causes various toxicological effects. As the injury of the gastrointestinal tract is an important aspect of DON toxicity, it is thus of great importance to characterize the effect of this mycotoxin on nutrient absorption in animals. The full effects of *Fusarium* mycotoxins are still not known. Therefore, the mode of action of *Fusarium* toxins in chickens need further research for being better understood. In our previous experiments, the obtained results clearly show that the electrogenic transport of glucose and glutamine is reduced by DON. Therefore experiments were conducted to test the effect of adding DON and wortmannin on the electrogenic transport of glucose. The present experiments have been performed to explain whether pharmacological inhibition of PI3 kinase by wortmannin (specific inhibitor of phosphatidylinositol-3-kinase, which is involved in the cascade of vesicle trafficking and insertion of transport proteins into the apical membrane) interferes with glucose transport.

Methods: Birds were fed the normal diet, which was free from mycotoxins. Intestinal segments of 5 chickens were taken from mid-jejunum; the tissue was mounted in Ussing chambers. After tissue stabilization, DON (10 ppm) or wortmannin (10 µM) were added to the mucosal side for 1 h. The treatment groups will be control, DON, wortmannin (as specific inhibitor of phosphatidylinositol-3-kinase, PI3), and DON + wortmannin (Pre-treatment of the tissues with wortmannin for 15 min and then DON for 45 min). After 1 h, 20 mmol/L of glucose were added to the mucosal side. The transepithelial short-circuit current (I_{sc}) and electrical tissue conductance (G_t) was recorded. Statistical differences between treatments were established by ANOVA and subsequent Duncan´s Multiple Probability values (P) of 0.05 or less were considered significant.

Results: Electrogenic Na+ transport via SGLT-1 induces a measurable current across intestinal epithelia. To assess SGLT-1 function, Isc was determined in the middle segment of the jejunum of chickens with the Ussing chamber technique. Addition of 20 mmol/L D-glucose to the luminal side of isolated jejunal tissues of control chickens produced an increase of Isc ($P < 0.01$). In contrast to the controls, the Isc was not significantly increased after glucose addition in the DON and in wortmannin group. The results indicate that DON decreased ($P < 0.05$) the glucose absorption in the absence of wortmannin. However, it had no additional effect on the glucose absorption in the presence of wortmannin.

Conclusion: These data suggest that the various effects of DON on nutrient transport is due to the ability of the tricothecene to inhibit protein synthesis and ascribed the functional consequences of acute mycotoxin action to their inhibitory action on PI3 kinase pathway which is required to maintain intestinal transport function. Whereas, the pharmacological inhibition of PI3 kinase markedly decreases the electrogenic transport of glucose.

Cytotoxicity and genotoxicity of the mycotoxins ochratoxin A and citrinin: Individual and combined effects

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Ochratoxin A (OTA) and citrinin (CIT) can co-occur in infected cereals. Both mycotoxins are known to exert nephrotoxicity in animals and also genotoxicity, such as induction of micronuclei. Treatment of cells with OTA produces mainly clastogenic effects, whereas CIT-induced micronuclei are predominantly centromere positive, indicative of an aneugenic effect [1].
In light of possible combined exposures to OTA and CIT, the present study now investigated over a range of concentrations the cytotoxic and genotoxic effects of binary mixtures and compared the effects to those caused by individual mycotoxins. To this end, cell viability was estimated by the neutral red uptake assay (NRU), and genotoxic activity of the mycotoxins was assessed by the micronucleus assay (MN) in V79 cells [2].
Cell viability was affected in a concentration dependent manner after exposure V79 cells for 48h, with significant reductions for single mycotoxins at 10 µM and more: The IC_{20} and IC_{50} values for OTA were 5 and 20 µM, those for CIT were 40 and 60 µM, respectively. Additive effects were observed in cytotoxicity assays when cells were simultaneously treated with concentrations of OTA and CIT which alone show low cytotoxicity. However, combinations at higher concentrations resulted in synergistic cytotoxic effects. Similar results were found in the genotoxicity assays with OTA and CIT: We observed an increase of MN with both single substances at slightly cytotoxic concentrations, and additive effects of the mixture at low micromolar concentrations. But at higher concentrations combined effects of OTA and CIT are clearly of synergistic nature.
In conclusion, the effects of a combined treatment with OTA and CIT depended on the tested concentrations. Lower concentration levels showed additive effects for the combination, whereas higher concentrations induced over-additive effects for cytotoxicity and genotoxicity.

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Effect of quercetin on the toxicokinetics of ochratoxin A in rats

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Introduction: Previous studies indicate that intestinal absorption of the nephrotoxic mycotoxin ochratoxin A (OTA) occurs mainly through passive diffusion of the undissociated form. However, several *in vitro* studies have shown that OTA is partly resecreted into the intestinal lumen by the multidrug resistance associated protein (MRP-2) and breast cancer resistance protein (BRCP). *In vitro* studies using Caco-2-cells have shown that polyphenols (quercetin, genistein, resveratrol) may impair OTA-efflux through competitive inhibition of the MRP-2, possibly resulting in an increased systemic availability of OTA (Sergent et al. 2005). Therefore, the aim of the present *in vivo* study was to investigate possible effects of quercetin on the toxicokinetics of OTA in rats.

Material and Methods: Eighteen growing male F344 Fisher rats with an average body weight of 200 g were allocated to two dietary treatments consisting of 1) a commercial, flavonoid-free balanced diet containing 10 mg OTA per kg (as fed) derived from inoculated wheat and 2) the same diet supplemented with 100 mg quercetin per kg. The animals were fed restrictively (~0.7 of *ad libitum* intake, 13 g/d) to avoid differences in OTA intake. Animals were kept in metabolism crates to facilitate total urine and faeces collection. After six days on trial, rats were euthanized and blood, liver, kidney, muscle and brain samples were taken from each animal. Faeces, urine and tissue samples were analysed for OTA and OTalpha by HPLC using fluorescence detection.

Results and Conclusions: Quercetin supplementation had no effect ($p>0.05$) on feed consumption (12.7 vs. 12.5 g/d), OTA-intake (137.1 vs. 130.0 µg/d), water intake (13.8 vs. 13.3) and body weight gain (1.9 vs. 1.7 g/d). Faecal and urinary excretion of OTA and OTalpha of rats was similar in both treatment groups. Concentrations of OTA in all tissues (Table) were not affected by quercetin supplementation as well. Based on the total excretion and tissue concentrations of OTA, it is concluded that the polyphenol quercetin has no impact on the toxicokinetics of OTA *in vivo.*

	OTA-flavonoid free (n=9)	OTA + Quercetin (n=9)
Plasma (µg/ml)	11.30 ± 2.10	10.90 ± 2.40
Kidney (µg/g)	1.44 ± 0.22	1.42 ± 0.15
Liver (µg/g)	1.12 ± 0.31	1.10 ± 0.14
Muscle (µg/g)	0.48 ± 0.09	0.53 ± 0.07
Brain (µg/g)	0.13 ± 0.02	0.14 ± 0.02

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Formation of glutathione adducts by 4-hydroxy-alternariol, a mammalian metabolite of alternariol

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Alternariol (AOH) is a major toxin produced by *Alternaria* fungi, which exhibits marginal acute toxicity but is genotoxic and mutagenic *in vitro*. Studies on the oxidative metabolism of AOH in liver microsomes [1] and in precision-cut rat liver slices [2] revealed the formation of four monohydroxylated metabolites with catechol structure. The toxicity of AOH might be enhanced by this pathway, as catechols can be metabolized to reactive (semi-) quinones, following adduct formation with proteins, DNA and glutathione (GSH). In addition, reactive oxygen species may be generated, causing GSH oxidation as well as modification of DNA bases and proteins.
The aim of the present study was to investigate the ability of AOH catechols to form adducts with GSH. We used 4-hydroxy (HO)-AOH, as this metabolite was accessible through chemical oxidation of AOH.
GSH adducts were analyzed by LC-DAD-MS, using UV spectra and MS^2 mass spectra for identification. Additionally, total GSH (including free GSH and glutathione disulfide) was determined photometrically with a modified method of Tietze [3].
The main product that could be detected after a 5-min incubation of 4-HO-AOH and GSH had UV maxima similar to the parent compound and *m/z* 578 with negative ESI-MS, which is the $[M-H]^-$ ion of a GSH monoadduct. Furthermore, typical fragmentation could be observed upon MS^2 analysis. Addition of $CuCl_2$ increased the amount of the monoadduct, and after addition of horseradish peroxidase and H_2O_2, the oxidized form of the monoadduct (*m/z* 576) could also be detected.
Incubation with 4-HO-AOH for 5, 30 and 60 min caused a time-dependent decrease of total GSH, which confirms GSH adduct formation of the catechol. In comparison with other catechols, e.g. estradiol catechols and 1,2-dihydroxynaphthalene, 4-HO-AOH was less potent.
In summary, 4-HO-AOH has been shown to react with GSH under cell-free conditions. It will be the subject of future studies to investigate the impact of 4-HO-AOH on cellular GSH.

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Silibinin pretreatment protects against ochratoxin A mediated apoptosis in cultured primary rat hepatocytes

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Silibinin is the major pharmacologically active compound extracted from the seeds of the milk thistle *Silybum marianum*. It is a widely used remedy and exhibits strong hepatoprotective activity against several liver toxins. Protective effects of silibinin on OTA- and LPS-mediated cytotoxicity in the liver have been described in perfused rat livers and isolated Kupffer cells and were related to inhibition of TNF alpha release [1]. Furthermore, antiapoptotic effects were described with silibinin on a human melanoma cell line [2].

Since OTA induces apoptosis in isolated rat primary hepatocytes [3], we investigated protective effects by silibinin on OTA- and LPS-mediated apoptosis and cytotoxicity in cultured rat hepatocytes. Primary rat hepatocytes were prepared by EDTA-perfusion method [4]. By treatment with 2.5 and 12.5 µmol/l OTA, cultured primary rat hepatocytes developed DNA-laddering after 24 to 36 h. LPS of 0.1 to 12.5 µg/ml had no apoptotic DNA-effects. Under the same conditions caspase-3 activity in hepatocytes increased under OTA within 12 to 24 h. In contrast, LPS incubation for 12 and 24 h did not alter caspase-3 activity.

Concentrations of (26, 52, 78, and 104 µmol/l) silibinin did not prevent OTA-mediated DNA-laddering after 24 and 36 h if applied 2 h prior to OTA, but the highest concentration of silibinin (≙130 µmol/l) showed complete inhibition of OTA-mediated apoptotic DNA-laddering and blocked caspase-3 activation of OTA under these conditions.

In summary, our results show differential effects on apoptosis in cultured primary rat hepatocytes by OTA (produces apoptosis) versus LPS (produces no apoptosis). Silibinin shows significant hepatoprotective effects against OTA-mediated apoptosis on cultured primary rat hepatocytes at high concentration if added up to 2 h prior to OTA [5].

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Zeranol induces oxidative DNA damage after metabolic activation with liver microsomes

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Zeranol (alpha-zearalanol, alpha-ZAL), used as a growth promotor (Ralgro®) in livestock in several non-EU countries, undergoes hydroxylation at the aromatic ring by liver microsomes of various species including humans [1] In addition to zearalanone (ZAN) as the major metabolite, the catechol metabolites 13-HO-alpha-ZAL, 15-HO-alpha-ZAL, as well as 13-HO-ZAN and 15-HO-ZAN were detected in all microsomal incubations. Human CYP1A2, which is highly expressed in the liver, is the most active CYP isoform for the aromatic hydroxylation of alpha-ZAL and ZAN. Catechol metabolites are frequently associated with the generation of reactive oxygen species (ROS) and subsequent oxidative damage of DNA, for which 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxo-dG) is a common biomarker.

In the present study, the propensity of catechol metabolites of alpha-ZAL and ZAN to cause the formation of 8-oxo-dG in isolated calf thymus DNA was determined using a LC-ESI-MS/MS method. To this end, alpha-ZAL was incubated with microsomes from human, rat, bovine and porcine liver as well as with human CYP1A2, and the incubations were extracted with ethyl acetate. The extract was analyzed with LC-MS and then added to a solution of calf thymus DNA in the presence of copper(II) ions and NADPH.

The formation of 8-oxo-dG could be demonstrated with each extract. The levels of 8-oxo-dG correlated directly with the extent of catechol formation, which increased from porcine to bovine to human to rat microsomes. It was concluded from the metabolic pattern that 15-HO-alpha-ZAL is more reactive than 13-HO-alpha-ZAL to form 8-oxo-dG. The most reactive metabolite was 15-HO-ZAN, which is the major metabolite of alpha-ZAL.

In conclusion, our study has shown that the catechol metabolites of alpha-ZAL are highly reactive and give rise to oxidative DNA damage *in vitro*. This finding warrants further investigations into the genotoxic potential of alpha-ZAL, which may constitute another biological activity in addition to the well-known estrogenicity.

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Ochratoxin A influences the expression of genes involved in renal pathophysiology in primary human proximal tubule cells

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Ochratoxin A (OTA), a mycotoxin produced by different *Aspergillus* and *Penicillium* species, is a ubiquitous contaminant of food and feed, which has both nephrotoxic and carcinogenic potential. The underlying changes of gene expression responsible for nephrotoxicity and cancerogenity, especially those after prolonged exposure to low concentrations of OTA, are still unknown. Therefore, the effect of nanomolar OTA concentrations on gene expression was analyzed in human kidney proximal tubule cells (RPTEC) in primary culture. Cells were incubated with 10 nM OTA for 14 days, RNA was isolated and gene expression was examined using different pathway specific RT² Profiler PCR Arrays. The PCR array data indicate that prolonged OTA exposure induces epithelial-to-mesenchymal transition by altering the expression of genes involved in this process (e.g. WISP1 (WNT1 inducible signalling pathway protein 1), FN1 (fibronectin 1)). Furthermore, OTA increases the expression of genes involved in inflammation (e.g. TNF-alpha) and in malignant transformation (e.g. APC (adenomatous polyposis coli)). Because of the strong up-regulation of WISP1 (fold induction 54,5) which belongs to the connective tissue growth factor family, the focus was laid on this gene as important profibrotic and oncogenic mediator and on TNF-alpha as proinflammatory cytokine. Evaluation of WISP1 and TNF-alpha gene expression via qPCR showed a significant up-regulation after stimulation with 10 nM OTA for 14 days, starting already after 2-7 days. In summary, a long-term exposure of human primary kidney cells with an OTA concentration detectable in human renal tissue changes the expression of genes that are involved in epithelial-to-mesenchymal transition, inflammation and malignant transformation and may act by influencing WISP1 signaling. Therefore OTA cause fibrotic changes in renal tissue and can contribute to tumorigenesis. These findings allow a new perspective on how OTA leads to kidney damage.

Inactivation pathways of the catechol metabolites of zearalenone

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Zearalenone (ZEN) is an estrogenic mycotoxin produced by *Fusarium* species. In a NTP carcinogenicity study, ZEN was found to induce hepatocellular and pituitary adenomas in mice. The mechanism of ZEN tumorigenicity is yet unknown and may be related to the hormonal activity. However, another possible mechanism may involve catechol metabolites of ZEN, i.e. 13- and 15-HO-ZEN, which are readily formed upon incubation of ZEN with hepatic microsomes from humans and various other mammalian species [1].The phase I metabolism of ZEN resembles that of the steroidal estrogen estrone (E1), which also forms two catechol metabolites, 2- and 4-HO-E1. Evidence is increasing that these catechols contribute to the carcinogenicity of E1 by acting as genotoxins after further activation to quinones [2].

It was the aim of the present study to examine possible inactivation pathways of the two ZEN catechol metabolites. Therefore, their methylation by catechol-O-methyltransferase (COMT) and their formation of adducts with N-acetylcysteine (NAC) was studied in comparison with the E1-catechols. For methylation, the catechol metabolites were incubated with human liver cytosol contained COMT and S-adenosyl-L-methionine. NAC adducts were generated by incubating the catechols with NAC. The products were analyzed by LC-ESI-MS.

Only one methylated metabolite was formed from each ZEN catechol with similar COMT activity of about 35 pmol/min/mg protein. Likewise, 4-HO-E1 gave rise to one methylation product with an activity of about 95 pmol/min/mg protein. In contrast, two methylation products were formed from 2-HO-E1 with about 10-fold higher activity. The extent of mercapturic acid formation was similar for the two ZEN catechols, but with higher yield than observed for the E1 catechols under the same conditions.

Our results suggest that the catechols of ZEN are less efficiently inactivated by COMT than the catechols of steroidal estrogens. This may be of toxicological relevance, because more catechol metabolites of ZEN should be available for redox cycling and subsequent oxidative damage. The extensive formation of NAC adducts underlines the potential of the ZEN catechols and their oxidation products to react with biomolecules.

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Uptake and metabolism of ergot alkaloids in human cells

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Ergot alkaloids are known as toxic secondary metabolites produced by fungi of the species *Claviceps*. Since the mediaeval times ergot poisoning is known to cause several effects, today known as ergotism. The occurrence in various grain products, especially based on rye, even after improvements of milling technology, reflects the necessity to evaluate the impact of ergot alkaloids on the human body.
Literature data show the evidence of an accumulation effect of ergot alkaloids in human primary cells and even in the human body *in vivo*. Besides this accumulation effect in cell lines it was the aim of our study to evaluate the metabolism by establishing a suitable *in vitro* model for ergot alkaloid metabolism studies in cell culture. HepG2 and HT 29 cells were used for this research. Our data could clearly demonstrate an accumulation effect in cell lines, as it is described for primary cells. The substance uptake in the cell interior increased in a time depending manner with a maximum of more than 1000 fold of the initial concentration in the cell media after six hours. Also a difference between -ine and -inine form was detectable for both used peptide ergot alkaloids. Therefore a very high accumulation of the used substances was verified. A third model compound, ergometrine did not show any uptake in human cells and no metabolites were detectable.
The data concerning uptake of ergot alkaloids were underlined by visualizing the substances using fluorescence microscopy. For the first time a simple approach, using the fluorescence properties of the ergot alkaloids without any labelled antibodies, could clearly show the uptake of the substances in human cells by a green fluorescence in the cytosol.
Besides this aspect, several metabolites were formed under *in vitro* conditions for the used model substances ergotamine and ergocristine. Using high resolution mass spectrometry (FTMS) a variety of metabolites were detectable in HT-29 and HepG2 cells including known hydroxy-metabolites as well as a new unknown metabolite. A postulated structure is presented in this work.
Our results clearly show that especially HT-29 cells serve as a very effective model for the analysis of ergot alkaloid metabolites *in vitro*.

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Reaction of intestinal epithelial cells (IPEC-J2) to deoxynivalenol in dependence of apical or basolateral application

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Background and aims: Deoxynivalenol (DON) is a cereal-contaminating mycotoxin, produced by fungi of the species *Fusarium graminearum* and may cause health risks for animals and humans. The first prominent barrier for nutritional toxins as DON is the intestine, which is in contact with the mycotoxin from the mucosa protected luminal side (apical exposure), as well as from basolateral side, where the absorbed toxin reaches the enterocytes via the blood stream. In the present study, we examined the impact of the DON application route on epithelial cell viability and intestinal barrier integrity.
Methods: A non-transformed intestinal porcine epithelial cell line (IPEC-J2), cultured on permeable membrane inserts, served as polarised *in vitro* model for the intestinal barrier. The effects of low (200 ng/ml) and high (2000 ng/ml) DON concentrations, exposed weather from apical or basolateral side on confluent cell layers were analysed for up to 72 h. Cell number was counted by DAPI stained nuclei. Proliferation was detected by BrdU-incorporation on single cell level by immuncytochemistry. Cell cycle phases were analysed by FACS of propidium iodide stained DNA. Induction of apoptosis was measured by caspase-3 activity in a luminescence assay. Effects of DON on tight junctions were characterized by TEER measurements, immunfluorescence staining for ZO-1 and claudin-3 as well as western blotting for claudin-3.
Results: An application of the low DON dose (200 ng/ml) showed no effect on the enterocytes, independently of direction or investigated assay. Also the high DON dose of 2000 ng/ml applied on the apical cell side was without an effect on enterocytes in all analysed assays. In contrast, the same high DON concentration given for 72 h on the basolateral side resulted in a breakdown of the integrity of cell connections, as well as a reduced expression of the tight junction proteins ZO-1 and claudin-3. Furthermore the cellular viability was reduced significantly. The decreased cell number was accompanied by an increase in BrdU-incorporation. Caspase-3 mediated apoptosis occurred early after 6 h incubation. Cell cycle analysis revealed an increase in DNA fragmentation, decrease in G0/G1 phase and slight increase in G2/M cell cycle phase.
Conclusions: The impact of the mycotoxin deoxynivalenol on the intestinal epithelial barrier is dependent on the route of application. The epithelium appears to be rather resistant towards apical (luminal) DON application whereas the same dose from basolateral (blood stream) severely undermines cell viability and barrier integrity.

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Cellular responses of fish cell lines to deoxynivalenol (DON) and zearalenol (ZEN)

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Contamination of animal feeds with mycotoxins is a major problem for fish feed mainly due to their inappropriate production and storage. Globally distributed and environmentally important mycotoxins are deoxynivalenol (DON) and zearalenone (ZEN) which are secondary metabolites of *Fusarium* fungi contaminating corn and cereal cultures in agricultural used regions. Mycotoxin-contaminated fish food has already been shown to induce changes in swimming behaviour and to impair growth of fish, but DON and ZEN have not yet been evaluated for their toxic potential in fish in detail. Thus, in initial experiments, the cytotoxic effects of DON and ZEN on five permanent cell lines established from several fish species were evaluated. Similar to research that has up to now concentrated on mammalian cell cultures DON and ZEN also showed cytotoxic effects on fish cell cultures. Furthermore, the involvement of oxidative stress in the cytotoxic mechanisms was investigated by using different assays. From these experiments it can be concluded that mycotoxins, such as DON and ZEN, exert detrimental effects on fish.

Reactivity of patulin: Influence of glutathione and glutathione-S-transferase on DNA adduct formation

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Due to its electrophilic structure, the mycotoxin patulin (PAT) reacts readily with nucleophiles, like glutathione (GSH), whose metabolic conjugation represents an universal detoxification. Thus, the mutagenic potential of PAT in cultured mammalian cells at submicromolar concentrations despite a large excess of the major intracellular nucleophile GSH raises the question of its molecular origin. Since co-incubation with thiols increases the reactivity of PAT towards amines, PAT might require activation by GSH in a glutathione-S-transferase (GST)-dependent or -independent way prior to reaction with DNA bases.
Therefore, cell-free reactions of PAT with adenine (Ade), adenosine (As) or guanosine (Gs) in the presence and absence of equimolar concentrations of GSH were carried out. The influence of GST on adduct formation was investigated using rat liver and mouse lymphoma cell cytosol each characterized regarding their GSH content and GST activity. Reaction products were separated by means of HPLC and identified by UV/Vis and MS/MS. Besides published PAT-GSH adducts, several PAT-Ade, PAT-As and PAT-Gs adducts were detected in the presence and absence of GSH. In addition, reaction products exhibiting a mass to charge ratio and fragmentation pattern suggesting mixed PAT-GSH-Ade, PAT-GSH-As or PAT-GSH-Gs adducts were detected. By incubation of PAT with cytosolic GST it was demonstrated that the formation of PAT-GSH adducts was accelerated compared to inactivated GST. Thus, we demonstrated the reactivity of PAT towards the DNA base Ade and the nucleosides As and Gs and the participation of GSH and GST in adduct formation for the first time.

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Ochratoxicosis on hematological and blood chemistry profiles in ducks

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Ochratoxicosis adversely affect to animal production particular swine and poultry industry. Poultry feed reportedly prone to be contaminated with mycotoxin. In poultry production, ochratoxicosis may reduce growth rate, increase susceptibility to diseases by reducing immune response as well as increase mortality rate (Verma et al., 2004). However, few studies of ochratoxicosis in duck are available. Therefore, aims of this study were set to observe the haematological, blood chemistry profiles and pathological change in duck following chronic exposure of low doses of ochratoxin A (OTA) by feeding.

Total of 48, one-day old, ducks (White Cherry Valley) were pooled and equally divided to 4 groups. After 1 week old, they were fed with OTA contaminated feed at concentration of 0, 50, 125 and 250 µg/kg for 6 weeks. Blood samples were taken from jugular vein at 2, 4 and 6 weeks after start feeding. Haematological profile was determined including Pack cell volume (PCV), Hemoglobin (Hb), Plasma protein (PP), Red blood cell (RBC), White blood cell (WBC) and differential count (%) of; Heterophil, Eosinophil, Basophil, Lymphocyte, Azurophil, and Monocyte. Serum collected samples were quantified for three blood chemistry profile; Serum glutamic oxaloacetic transaminase (SGOT), creatinine kinase (CK) and uric acid. Postmortem examinations were performed on each duck at the end of study.

Feeding with low dose of OTA in the present study showed no effect on red blood cell production. For total white blood cell, when considering within the high contaminated feeding groups, WBC has tendency to decline by time of exposure especially lymphocytes and eosinophils count. None of blood chemistry profiles were significantly different after OTA ingestion. However, variation value of uric acid in some groups may result from kidney damaged by OTA (as seen in the microscopic level).

In conclusion, variation fashion of hematological and serum blood chemistry levels changed among different low concentrations of OTA contamination are noted. Consequently, neither of hematological nor serum blood chemistry profiles could specific indicate the ochratoxicosis in duck. However, OTA contamination has tendency to suppress the immune system in duck even low-dose of exposure.

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The level of zearalenone and its metabolites in urine samples in patients with prostate cancer and benign prostate hyperplasia

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The benign prostate hyperplasia (BPH) and prostate cancer are the most often over 50 man's diseases. The latter can have latent and gradual development, namely at older men. The prostate problems may also occur at younger patients. During its initial phase of development (when focused only within the gland), prostate cancer gives no symptoms. It sometimes accompanies the mild prostate hyperplasia and invokes lower urinary tract symptoms (LUTS). Mainly disturbances of estrogens and androgens conversion, as well as nutritional errors are counted as etiological factors. The food products made of such materials as wheat, oats, rye, sorghum, and namely maize, may contain dangerous mycotoxins, among which zearalenone (ZEA) is one of the secondary metabolites. Zearalenone is produced by some *Fusarium* strains. The compound has estrogenic properties and is responsible for disturbances associated with hormonal and reproduction systems functioning. Prostate is hormone-dependent organ. Androgens (mainly testosterone) and elevated concentrations of estrogens regulate its functions and contribute to its hyperplasia. Thus, it can be supposed that elevated levels of estrogenic zearalenone is a factor that affects the development and tumor changes of prostate as well as adjacent organs, e.g. bladder.

The aim of the study was the evaluation of the content of zearalenone and its metabolites - alpha and beta zearalanol (alpha- ZAL and beta- ZAL) as well as alpha and beta zearalenol (alpha- ZOL and beta- ZOL) in urine at patients with prostate disorders.

Zearalenone and its metabolites were determined with the HPLC-MS/MS (Applied Biosystems) method. The extract of zearalenone from samples was cleaned-up with ZearalaTest™ columns.

The research has been carried out in cooperation with the Urology Department of the Biziel's Hospital in Bydgoszcz. The samples of urine were collected from 121 men aged between 40 and 80, with 77 being the samples taken from the patients suffering from prostate cancer, 35 from the patients suffering from benign prostate hyperplasia and 3 from the patients who had prostate removed during the operation of the bladder. The control examinations were performed using urine from 6 men of the same hospital department, while they did not complain about any prostate disorders.

The average concentration of ZEA at patients suffering from prostate cancer, benign prostate hyperplasia, and those who underwent prostate removal during the surgery of the bladder and in control group was respectively: 0.200 ng/ml (max 8.345 ng/ml), 0.053 ng/ml (max 0.158 ng/ml), 0.046 ng/ml (max 0.061ng/ml), and 0.082 ng/ml (max 0.165 ng/ml). The average concentration of ZEA in the urine from all the patients with prostate disorders was 0.189 ng/ml (max 8.345 ng/ml).

Samples also revealed the ZEA metabolites. Urine from prostate cancer patients contained: alpha-ZOL 0.057 ng/ml (max 0.314 ng/ml) and beta-ZOL 0.065 ng/ml (max 1.420 ng/ml); that from patients with benign prostate hyperplasia: alpha-ZOL – 0.054 ng/ml (max 0.158 ng/ml), and beta-ZOL – 0.054 ng/ml (max 0.574 ng/ml), whereas from patients with surgically removed prostate: 0.067 ng/ml (max 0.136 ng/ml), and 0.039 ng/ml (0.086 ng/ml). Urine from all patents contained slight amounts of alpha-ZAL and beta-ZAL.

The results are a part of the study included in the project no NR12001706.

T-2 toxin alters host-pathogen interactions of *Salmonella* Typhimurium in pigs

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T-2 toxin is a very stable mycotoxin produced by *Fusarium* spp. which often ends up in the food and feed chain. It poses a threat to human and animal health, especially to pigs which appear to be one of the most sensitive species (Hussein and Brasel, 2001). Besides mycotoxins, *Salmonella* Typhimurium infections are a major issue in swine production. Therefore, the aim of the presented study was to investigate the effects of T-2 toxin on the course of a *Salmonella* Typhimurium infection in pigs.

We conducted an animal trial in which 3-week-old *Salmonella*-free piglets were randomized into three groups of 5 piglets. During 23 days, they received *ad libitum* blank feed (control group), feed contaminated with 10 µg/kg T-2 toxin (10 ppb group) or feed contaminated with 100 µg/kg T-2 toxin (100 ppb group). The pigs were orally inoculated with 2×10^7 colony forming units (CFU) of *Salmonella* Typhimurium. Five days after inoculation, the pigs were humanely euthanized and samples of intestinal contents and tissues were taken for bacteriological analysis. Compared to the control group, the 10 ppb and 100 ppb group showed lower numbers of *Salmonella* Typhimurium in jejunum, cecum ($p < 0.05$) and colon tissue, and cecum ($p < 0.05$) and colon contents. These results show that the presence of low and in practice relevant concentrations of T-2 toxin in the feed reduces the colonization of *Salmonella* Typhimurium in the intestine. To elucidate the cause of this reduced colonization, we investigated the effects of T-2 toxin on the host-pathogen interactions of *Salmonella* Typhimurium and on the gene expression of the bacterium, *in vitro*. We showed that very low concentrations of T-2 toxin (1-10 ng/ml) promote the susceptibility of porcine alveolar macrophages (PAM) and intestinal porcine epithelial cells (IPEC-J2) to *Salmonella* Typhimurium invasion, but not to intracellular proliferation of the bacterium. Furthermore we demonstrated that T-2 toxin promotes the translocation of *Salmonella* Typhimurium over an IPEC-J2 monolayer, at concentrations (1-5 ng/ml) that did not exert a notable effect on porcine cell viability and transepithelial electrical resistance (TEER). These *in vitro* findings show that T-2 toxin might result in enhanced colonization of *Salmonella* Typhimurium in pigs. However, microarray analysis showed that T-2 toxin, at a concentration as low as 5 ng/ml, reduces expression of *Salmonella* Typhimurium motility genes *flgL*, *flgM* and *flgK*. It also causes a general downregulation of the metabolism of *Salmonella* Typhimurium, which could lead to a decreased colonization of the bacterium. Probably, *in vivo*, T-2 toxin influences the gene expression of the bacterium before the cell-mediated effect takes place.

In conclusion, T-2 toxin significantly affects the gene expression and host-pathogen interactions of *Salmonella* Typhimurium resulting in a reduced colonization of the bacteria in pigs.

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T-2 toxin: Metabolism in human cells

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The type A trichothecene T-2 toxin is a common contaminant of food and feed. The various fungi of the *Fusarium* species producing T-2 toxin infect mainly cereals like wheat, corn and oat on field or during storage. Knowledge about its toxicity and metabolism in the human body are important since T-2 toxin can still be detected in processed food samples. After consumption, several acute as well as chronic toxic effects were observed in humans and animals. To study the cytotoxicity and the metabolism of T-2 toxin *in vitro*, cell culture studies using cell lines and primary cells derived from human organs are a suitable model system.
In literature, various metabolites of T-2 toxin are described in animal studies. Phase I modifications like hydrolysis, hydroxylation, and oxidation are key steps in the metabolism with HT–2 toxin being the main metabolite besides neosolaniol, T-2 triol, and T-2 tetraol. Further metabolites resulting from phase II reactions are glucuronide conjugates which could also be traceable *in vivo*.
The aim of this study was to compare the metabolic activity of a cell line derived from human colon carcinoma cells (HT-29) and primary human renal proximal tubule epithelial cells (RPTEC). Cells in primary culture are directly isolated from the corresponding tissue and are therefore more comparable to the *in vivo* situation. After incubating the cells with T-2 toxin for 24 and 48 hours exact mass measurements using LC-FTMS could reveal the formed metabolites in comparison with reference compounds. Several metabolites were detectable after the chosen incubation periods, namely HT-2 toxin and neosolaniol in both cell types and even glucuronide conjugates in HT-29 cells.
Furthermore, cell cycle analysis in RPTEC could confirm the toxic effect of T-2 toxin on human cells. The formation of a subG1 peak was used as approval of apoptosis induced by T-2 toxin.

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Trichothecene-glucuronides: Formation and analysis in liver microsomes

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The trichothecenes T2-toxin (T-2) and HT2-toxin (HT-2) are important contaminants in cereals like oat, wheat or corn. They are produced by a variety of *Fusarium* fungi and a number of toxic effects are ascribed to them.

Several *in vitro* and *in vivo* studies were conducted to examine the metabolism of T-2 and numerous metabolites were identified. The spectrum and amount of metabolites varied, depending on the investigated species. Typical phase I reactions for the metabolism of T-2 comprise hydroxylation, hydrolysis or deepoxidation. The deacetylation product HT-2 was found to be one of the major metabolites of T-2. However, T-2 and its metabolites can also be conjugated in typical phase II reactions to polar moieties like glucuronic acid.

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

Liver microsomes are not only used for the synthesis of glucuronides of xenobiotic substances but they are also widely employed as an *in vitro* model for the investigation of metabolism. Therefore the formation of T-2 glucuronides was studied using microsomes prepared from livers of different species. Differences in the glucuronidation of T-2 depending on the animal species were observed.

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Effect of increasing concentrations of T-2 toxin on laying hens

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Introduction: T-2 toxin is one of the most toxic mycotoxins belonging to the type A trichothecenes. It is produced by several *Fusarium* species, like *F. acumimatum*, *F. poae* and *F. sporotrichoides*. The ingestion of mycotoxin contaminated feed may have adverse effects on the performance and health of farm animals. In laying hens, dietary concentrations of 1-20 mg/kg T-2 toxin were reported to cause decreased feed intake, egg production, egg shell thickness and oral lesions [1]. However, dose response studies using recent genetics of laying hens are scarce. Thus, the aim of the present study was to determine the effect of an exposure of more practically relevant dietary T-2 toxin concentrations on the performance and health of laying hens, and on egg quality.

Methods: A total of 36 laying hybrids (Lohmann Brown, 31 week old) were assigned randomly to 4 groups with 9 hens each. The study was designed as a balance experiment. Thus, hens were kept individually in balance cages and were fed restrictively (45 min feed allowance, two portions à 45 g) with experimental diets containing 0, 0.27, 1.05 and 4.10 mg T-2 toxin/kg. T-2 toxin (kind gift of Prof. H.-U. Humpf, Westfälische Wilhelms-University, Münster) was blended into the diets as crystalline solid dissolved in ethanol. Laid eggs, egg weight and feed consumption were recorded daily. Body weight was recorded weekly. Concerning oral lesions, the beaks were evaluated regularly (3 steps score). All hens were slaughtered after 21 days of feeding the experimental diets and organ weights were recorded. The data were evaluated by ANOVA, means were compared by Fischer-LSD test ($p<0.05$) with exception of the non parametric oral lesion data which were evaluated by Mann-Whitney-U-test.

Results: The T-2 toxin contamination of the feed did not result in significant differences in the abundance of oral lesions which were observed in hens of all feeding groups. Similarly, neither the performance as indicated by the egg mass production and feed conversion, nor the egg quality specified by yolk, albumen and egg shell weight were affected by the treatment. The relative weight of the crop of hens was significantly affected by the presence of T-2 toxin in feed. However, no dose response relationship could be observed. All other organ weights were not affected.

Conclusions: Under the conditions of this study no significant effect of practically relevant dietary T-2 toxin concentrations on the performance of laying hens and on the egg quality could be observed. The significant T-2 toxin effects on the crop weight can not be explained and require further elucidation to clarify this result.

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Intestinal adaptation in chicken to chronic mycotoxin challenge involves specific reduction of unit absorptive surface while increase in length

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Various mycotoxins are known to deteriorate small intestinal mucosa in terms of morphology and absorptive function. In case of low levels of some mycotoxins (for instance trichothecenes), this deterioration of mucosa has however not been noted to affect bird performance. Present trial was therefore conducted to study the development of mycotoxin-induced morphological changes in small intestine and their effect on absorptive and digestive functionality of the organ during exposure to aflatoxin B_1 (AFB_1) and deoxynivalenol (DON). For this purpose, Ross male chicks at 7 d age were reared under basal, low AFB_1 (0.07 mg AFB_1/kg), high AFB_1 (0.75 AFB_1 mg/kg), low DON (1.4 mg/kg), or a high DON (14 mg/kg) diet. Increasing levels of both the AFB_1 and DON caused a linear decrease in the unit weight of small intestine. This reduction in unit weight of small intestine was found to be due to decrease in villus height and was also found to progress from proximal to distal small intestine with increase in the length of exposure. This reduction in absorptive surface was accompanied with altered electrophysiology in jejunum of birds under DON diets but not in case of birds under AFB_1 diets. Interestingly, a seemingly compensatory increase (linear effect) in the length of small intestine was noted to occur with increasing levels of both the DON and the AFB_1. Thus, nominal improvements in retention of macro nutrients were noted under mycotoxin diets. Contrary to the digestibility of macro nutrients, the transfer of DON and metabolites to systemic circulation was decreased in the birds reared under high DON diet. These data indicate that unit absorptive function of small intestine in chicken may decrease in a specific fashion depending upon the type of mycotoxin present in the diet. However, chickens are also able to compensate the reduced unit absorptive functionality by modulating the length of small intestine.

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