SESSION 1:

FOOD SAFETY, TOXICOLOGY AND UTILIZATION OF MYCOTOXIN-CONTAMINATED GRAIN

Chairperson: Jim Pestka

QUANTIFICATION OF THE *TRI*5 GENE, EXPRESSION AND DEOXYNIVALENOL PRODUCTION DURING THE MALTING OF BARLEY.

Anuradha Boddeda[1], Paul Schwarz[2] and Charlene E. Wolf-Hall[1*]

[1]Department of Veterinary and Microbiological Sciences, North Dakota State University, Fargo, ND; and [2]Department of Plant Sciences, North Dakota State University, Fargo, ND
[*]Corresponding Author: PH: (701) 231-6387; Email: Charlene.Hall@ndsu.edu

ABSTRACT

Barley quality and safety is affected by *Fusarium* both in the field and during post-harvest processes. *Fusarium* strains can survive, grow and produce mycotoxins during malting. We evaluated percentage of *Fusarium* infection (FI), and deoxynivalenol (DON) concentration in three, raw barley samples (high quality, naturally-infected, *F. graminearum* inoculated barley) during various stages of malting. We also applied real-time PCR and real-time reverse transcriptase PCR (real-time RT-PCR) methods to quantify the *Tri*5 DNA concentration and expression respectively in the barley samples. We observed that FI significantly ($P < 0.05$) increased during the germination stage of malting in all the barley samples. Temperatures of 49°C and higher during kilning reduced the FI in high quality barley samples, but for inoculated samples more than 60°C during kilning was needed to reduce *Fusarium* infection. The average *Tri*5 DNA was found to be in the range of 0 to 3.9 ng/50 mg, 0.06 to 109.79 ng/50 mg and 3.38 to 397.55 ng/50 mg in malted high quality, inoculated and infected barley samples respectively. Strong gene expression (*Tri*5) in naturally infected barley samples was found during 3rd day of germination, however very low amounts of gene expression were observed in high quality and inoculated barley samples during malting. Deoxynivalenol was found to be present even at high kilning temperatures as DON is heat stable. The average DON concentration was found to be in the range of 0 to 0.13 µg/g, 0 to1.09µg/g and 1.53 to 45.86 µg/g during malting in high quality, inoculated and infected barley samples respectively. Overall, the last two days of germination and initial stages of kilning were peak processing stages for FI, *Tri*5 gene production, *Tri*5 gene expression and DON production.

HIGH-THROUGHPUT HOMOGENIZATION OF GRAIN SAMPLES FOR DON TESTING.

D.M. Reaver and D.G. Schmale III*

Virginia Polytechnic Institute and State University, Blacksburg, VA, 24061
*Corresponding Author: PH: (540) 231-6943; Email: dschmale@vt.edu

ABSTRACT

Concerns about deoxynivalenol (DON) continue to mount, and there is a growing need to develop new tools and techniques to enhance the speed, capacity, and uniformity of DON testing services in the United States. Tens of thousands of wheat and barley samples associated with USWBSI research projects are processed by DON testing labs every year. Many of these samples consist of 100 g kernel lots, and they must be cleaned, milled, and sieved before DON is extracted and quantified. The processing of a high number of grain samples in such a manner is extremely laborious and costly. We developed a rapid and affordable high-throughput homogenization protocol for DON testing that can homogenize twelve grain samples weighing from 0.1 to 2.5 g in as little as ten seconds. A Biospec MiniBeadBeater-96 ™operating at 2100 oscillations per minute was used to homogenize grain samples in individual 7 mL HDPE vials containing 13.7 mm chrome balls. Grain samples were homogenized into a fine flour of nearly uniform particle size, and DON extractions were conducted in the same vials that were used for the homogenization of the samples. The extraction solvent containing DON was passed through a clean-up column, and a measured fraction of the flow-through was dried down using a nitrogen evaporator at 55C. DON samples were derivatized using TMSI and quantified using a GC/MS operating in a SIM/SIM scan mode for target and reference ions of DON. Over 800 grain samples originating from single inoculated or non-inoculated (control) wheat spikes from southern uniform FHB greenhouse trials in AR, NC, and VA were processed for DON testing using this new methodology. High-throughput homogenization protocols may assist in providing affordable and timely DON testing services for USWBSI-associated research projects in the future.

ANALYSIS OF DEOXYNIVALENOL, MASKED DEOXYNIVALENOL AND *FUSARIUM GRAMINEARUM* PIGMENT IN GRAIN CULTURES USING A NEW LC-UV/MS METHOD.

Sasanya, J.J.[1], Hall, C.[1,2*] and Wolf-Hall, C.[1,3]

[1]Great Plains Institute of Food Safety, North Dakota State University (NDSU), Fargo, ND; [2]Department of Cereal and Food Sciences, NDSU, Fargo, ND; and [3]Department of Veterinary and Microbiological Sciences, NDSU, Fargo, ND
[*]Corresponding Author: PH: (701) 231-6359; Email: clifford.hall@ndsu.edu

ABSTRACT

The presence of the mycotoxin deoxynivalenol (DON, 3,7,15-trihydroxy-12,13-epoxytrichothec-9-ene-8-one) in grains presents a food safety risk. Also, DON may conjugate with sugars resulting in masked mycotoxins such as deoxynivalenol-3-glucoside (DON-3-glucoside) which may be metabolized *in vivo* to DON thus increasing the risk. Such masked mycotoxins and the potentially toxic *Fusarium* pigment are not routinely analyzed in grains. To promote continued understanding of the presence of masked mycotoxins in grains and their coexistence with DON, we analyzed rice and wheat culture samples inoculated with different *Fusarium* strains, using a new liquid chromatography (LC) - mass spectrometry (MS) method. We also quantified the *Fusarium* pigment. Grain samples cultured for 14 days were extracted by centrifugation with methanol:methylene chloride (50/50, v/v) followed by cleanup through C18 columns. Elution solvents included methanol, water, acetonitrile and acetic acid before analysis using LC-UV/MS. An isocratic mobile phase (70% methanol) was used. The DON average in rice culture was 15.2 (± 41.0) mg/kg while DON-3-glucoside averaged 5.4 (± 8.6) mg/kg in rice. Neither DON nor DON-3-glucoside were observed in wheat culture samples. The pigment averaged 142.2 ±256.3 mg/kg in wheat and 7.1±126 mg/kg in rice culture samples. Therefore, we report here how analytical tools such as this new LC-UV/MS method can be used to quantify masked and parent mycotoxins in rice plus potentially toxic pigment in rice and wheat culture for risk assessment studies. The coexistence of DON with DON-3-glucoside in grain cultures such as rice spiked with *Fusarium graminearum* emphasizes the conjugation of DON to form masked mycotoxins hence the need to regularly analyze grains for both parent and masked mycotoxins. Such studies can be optimized to further explore ways of producing and isolating masked mycotoxins in culture since standard masked mycotoxins are not commercially available. Potentially toxic pigments can also be studied further.

PRODUCTION AND PURIFICATION OF DEOXYNIVALENOL FROM RICE CULTURE AND ANALYSIS USING LIQUID CHROMATOGRAPHY-ULTRAVIOLET-MASS SPECTROMETRY.

Sasanya, J.J.[1], Hall, C.[1,2*] and Wolf-Hall, C.[1,3]

[1]Great Plains Institute of Food Safety, North Dakota State University (NDSU), Fargo, ND; [2]Department of Cereal and Food Sciences, NDSU, Fargo, ND; and [3]Department of Veterinary and Microbiological Sciences, NDSU, Fargo, ND
[*]Corresponding Author: PH: (701) 231-6359; Email: clifford.hall@ndsu.edu.

ABSTRACT

Mycotoxins such as trichothecenes represented by deoxynivalenol (DON) present a major global food safety problem with associated economic implications. Thus toxicological, analytical and detoxification studies relevant to industry and regulatory agencies are priority. Such studies require relatively large quantities of pure mycotoxins such as DON. Here we present a new method were we produced and purified (96-99%) large quantities of DON (403 μg/g) from rice (85g) inoculated with *Fusarium graminearum* ($3x10^7$ spores per ml) and incubated for 4 days at 30 °C. Purification was achieved using a combination of silica gel (32 g), alumina (7.2 g) and celite (4.8 g) in a glass column. This followed extraction by high speed centrifugation using methanol:methylene chloride (50/50, v/v). High recovery rates (100 ± 9.9%; CV=0.1) were also recorded. Elution of the mycotoxin was done using methanol, acetonitrile, water and acetic acid (60:30:10:0.1, v/v). Analysis of DON was done using thin layer chromatography and liquid chromatography connected to ultraviolet and mass spectrometer detectors. These findings present a new method to culture and purify DON in bulk using relatively cheaper clean up column followed by analysis using chromatographic and mass spectrometric techniques. Such findings also support toxicological, analytical and detoxification studies pertaining DON.

DEOXYNIVALENOL MEASUREMENT: SOURCES OF ERROR AND SAMPLING RECOMMENDATIONS.

Paul Schwarz[1*], Yanhong Dong[2] and Ruth Dill-Macky[2]

[1]Department of Plant Sciences, North Dakota State University, Fargo, ND 58105; and
[2]Department of Plant Pathology, University of Minnesota, St Paul, MN 55108
[*]Corresponding Author: PH: (701) 231-7732; E-mail: Paul.Schwarz@ndsu.edu

ABSTRACT

For any type of analysis there is measurement error that is composed of various factors such as sampling, sample preparation, and the analytical instrument itself. In the determination of deoxynivalenol (DON) by gas chromatography (GC), sample preparation steps include grinding, extraction, clean-up and derivatization. Factors influencing derivatization are probably most critical, and have greater influence than instrument variability. Inter-laboratory check samples of ground wheat and barley are periodically analyzed by the USWBSI funded laboratories as a means of assuring consistency of results. The coefficients of variation (CV) on these analyses typically range from 5 to 15%, which is considered acceptable for analytical work in the mg/kg (ppm) range. As an example, the expected analytical range in DON results with a CV of 10% for a sample at 1.00 mg/kg would be 0.89- 1.11 mg/kg. However, it must be remembered that much of the variability observed in DON levels in grain is related to the biology of the disease, rather than the chemical analysis. This follows as DON accumulation in grain results from a complex host-pathogen interaction which is subject to environmental variability. The production of DON, like visible symptoms of FHB, varies greatly from spikelet to spikelet, spike to spike, and environment to environment. Grain sampling greatly affects the accuracy of DON analysis, and the responsibility of providing a representative sample rests with the individual researcher. Sampling considerations include the collection of a representative samples from the experimental unit (plot, field), and then the reduction of this material to a representative sub-sample with a sample divider. The sample provided to the DON analysis laboratories should be no more than 100 g, and ideally around 20 g. This follows, as the grinding of 10,000 100g samples, as opposed to 20 g samples, requires an additional 21 days of labor. USWBSI recommendations on the sampling of grain for DON are posted at http://www.scabusa.org/pdfs/ptt/researchers_grain-sampling-protocols.pdf

DEVELOPMENT OF A MULTIPLEX REAL-TIME PCR ASSAY FOR RAPID DETECTION AND QUANTIFICATION OF *FUSARIUM* SPP. IN BARLEY.

D.J. Tobias[1*], A. Vashisht[1], A. Boddeda[1], C.E. Wolf-Hall[1] and P.B. Schwarz[2]

[1]Dept. of Veterinary and Microbiological Sciences, and [2]Dept. of Plant Sciences, North Dakota State University, Fargo, ND 58105
[*]Corresponding Author: PH: (701) 231-6386; Email: dennis.tobias@ndsu.edu

ABSTRACT

The persistence of trichothecenes in *Fusarium*-infected stored grains and in processed food poses a great risk to human health and animals. The ability to rapidly detect *Fusarium* species and monitor their distribution in collected wheat and barley grains across the state of ND is important due to the significant number of grain samples and the differences in the toxicity of these secondary metabolites. This can be accomplished rapidly using a polymerase chain reaction (PCR)-based detection of FHB-associated *Fusarium* species. Our objective is to develop a multiplex real-time PCR assay to identify and quantify pathogenic *Fusarium* species based on primer pairs derived from Intergenic Spacer (IGS) of rDNA unit sequences. The selected primers for species-specific detection showed amplification products of 123, 418, 462, 293 and 186 bp using positive controls (template DNA) which were derived from *F. graminearum* (NRRL R-6574), *F. avenaceum* (FRC# R-04608), *F. poae* (FRC# T-0487) *and F. sporotrichioides* (FRC# T-0348), respectively. Multiplexing (3 to 4-species) resulted in amplifications for species-specific detection using naturally-infected malting barley, Robust, with 0 and 2 ppm DON levels. Five picograms of fungal DNA were found to be enough to obtain a visible amplification product. For reliability, the multiplex real-time PCR assay will also test several isolates of each *Fusarium* spp. This high-throughput assay will help screen the malting barley samples and accurately assess the distribution of *Fusarium* spp. which are predominant in the region. Malting barley collections in 2005-2006 from 3 ND districts (and 1 MN district) will be utilized for future assays.

OZONE AS AN ANTIMYCOTIC AGENT IN MALTING BARLEY.

Tobias D.J.[1], C. Wolf-Hall[1*] and P.B, Schwarz[2]

[1]Department of Veterinary and Microbiological Sciences; and [2]Department of Plant Sciences, North Dakota State University, Fargo, ND 58105
[*]Corresponding Author: PH: (701) 231-6387; Email: charlene.hall@ndsu.edu

ABSTRACT

Fusarium spp. are known producers of important mycotoxins such as trichothecenes. The persistence of trichothecenes in infected stored barley grains and in processed food poses a great risk to human health and concern in the malting and brewing industry. At present, the only available effective control is testing and diversion or dilution. The effectiveness of ozone as an insecticidal fumigant in stored grains has been reported previously. The objective of this project is to evaluate the efficacy of gaseous ozone as an antifungal and antimycotoxin treatment for barley. Preliminary tests by gaseous ozone treatment (GOT) of pure *Fusarium graminearum* (FRC# R-06574) culture at 26 mg/g O_3 for 90 min. on the broth surface showed a detrimental effect on the morphological structure (non-branching and breakage of hyphae) and a 30% decrease in fungal biomass. The fungus was not totally eliminated probably due to the presence of sugar and nutrients in the broth allowing recovery and slow growth. In the present study, an extended treatment of 120 min at 26 mg/g O_3 were tested on five *Fusarium* spp. (*F. graminearum, F. culmorum, F. poae, F. sporotrichioides* and *F. avenaceum*) separately grown in PD broth. The same treatment was applied to malting barley before steeping and after 2 or 8 hrs steeping through a submerged gas sparger. We report on the effect of ozone on the growth and survival (FS) of five *Fusarium* spp., germinative energy (GE) and DON in malting barley.

ECONOMIC PERSPECTIVES OF GROWERS FACING THE CHALLENGES OF FHB AND DON.

Felicia Wu

Department of Environmental & Occupational Health, Graduate School of Public Health, University of Pittsburgh, 100 Technology Dr. Suite 560, Pittsburgh PA 15219
Corresponding Author: PH: 412-624-1306; Email: few8@pitt.edu

ABSTRACT

The economic problem of Fusarium Head Blight (FHB) and DON is extremely complex, yet can provide insight into how to focus research and development to prevent these conditions. We start with the guiding question: How can the economic perspective of wheat and barley growers help us decide how we should focus our research and initiatives? This talk describes four categories of economic consideration: 1) the sources of uncertainty and variability in wheat and barley production, FHB, and DON; 2) the cost-effectiveness of various control methods to reduce FHB and DON; 3) benefit-cost analysis of adopting FHB/DON control methods; and 4) the tradeoffs for growers planning to plant wheat or barley compared with competing crops such as corn.

SEX DIFFERENCES IN APPARENT ADAPTATION TO IMMUNOTOXICITY OF DEOXYNIVALENOL.

Xianai Wu[1], Marian Kohut[2], Joan Cunnick[3] and Suzanne Hendrich[1*]

[1]Food Science and Human Nutrition, [2]Kinesiology, and [3]Animal Science,
Iowa State University, Ames, IA 50011
[*]Corresponding Author: PH: 515-294-4272; Email: shendric@iastate.edu

ABSTRACT

Deoxynivalenol (DON) is a mycotoxin produced by *Fusarium graminearum* and *F. culmorum* commonly found in grains. We hypothesized that DON was immunotoxic in BALB/c mice, suppressing peripheral blood lymphocytes at 1 ppm but not at lesser doses.

Groups of 10 female and 10 male BALB/c mice were fed DON at 0, 0.25, 0.5, 1, and 2 ppm over 14 days and 28 days. Peripheral blood and single spleen cells suspension were stained with fluorescently labeled antibodies for CD4, CD19, CD8a and CD11b leukocyte cell surface markers. Flow cytometry was used to detect leukocyte phenotypes.

In peripheral blood, the percentage of T cytotoxic and B cells were inhibited in both sexes of BALB/c mice after 14 days of DON exposure, and toxic dose of DON varied by sex, whereas exposure to DON over 28 days did not inhibit these lymphocytes, compared with the control diet. Dietary DON did not influence hematology in males but red blood cells (RBC) at 0.5 and 1 ppm DON and hemoglobin (Hb) at all DON doses were suppressed in female mice by dietary DON over 14 days. The inhibition of RBCs by DON disappeared after 28 days compared with the control diet. The percentage of monocytes (CD11b+) was decreased in peripheral blood (at doses of 0.5-2 ppm DON) and spleen (2 ppm DON) only in BALB/c female mice after 28 days compared with control diet.

These results indicate that BALB/c mice adapted to most signs of DON immunotoxicity and hematotoxicity after 28 days. At this time, the percentage of monocytes was decreased in peripheral blood and spleen by as little as 0.5 ppm DON in female mice, suggesting that female sex hormones potentiate DON immunotoxicity in BALB/c mice.

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DOEHLERT MATRIX DESIGN FOR OPTIMIZATION OF THE DETERMINATION OF BOUND DEOXYNIVALENOL IN BARLEY GRAIN WITH TFA.

Bing Zhou[2], Yin Li[1], James Gillespie[1], Richard Horsley[1] and Paul Schwarz[1*]

[1]Department of Plant Sciences, North Dakota State University, Fargo, ND 58105; and [2]Department of Food Sciences and Nutrition, Zhejiang University, Hangzhou, 310029 P.R.China
[*]Corresponding Author: PH: 701-231-7732; Email: Paul.Schwarz@ndsu.edu

ABSTRACT

Fusarium Head Blight (FHB) is an impediment to barley production in many regions of the world. Tricothecene toxins, associated with FHB infected grain, particularly deoxynivalenol (DON) pose a serious threat to human and animal health. Recent research has suggested that a portion of the DON present on grain is bound and escapes detection through conventional determination. The objective of this study was to optimize a method for determination of non-extractable DON in barley grain using TFA. A Doehlert matrix design was performed to determine the optimal conditions for time, temperature and TFA concentration. These conditions were treatment with 1.25 N TFA in 86:14 acetontrile:water for 54 min at 133°C. Clean-up, derivatization and determination of DON by GC-ECD was as normal. Treatment of the test sample resulted in release of an additional 58% DON under the optimized conditions, and an increase of 9 to 88% in a set of verification samples.

EFFECT OF ENZYME PRETREATMENTS ON THE DETERMINATION OF DEOXYNIVALENOL IN BARLEY.

Bing Zhou[2], James Gillespie[1], Richard Horsley[1] and Paul Schwarz[1*]

[1]Department of Plant Sciences, North Dakota State University, Fargo, ND 58105; and [2]Biosystems Engineering and Food Sciences, Zhejiang University, Hangzhou 310029, People's Republic of China
[*]Corresponding Author: PH: 701-231-7732; Email: Paul.Schwarz@ndsu.edu

ABSTRACT

The impact of particle size and enzymatic treatment was determined on the quantification of deoxynivalenol (DON) in FHB-infected barley samples. Particle size significantly affected DON determination in eight of ten samples analyzed. Significance of the barley sample x particle size interaction demonstrated that samples did not respond uniformly to the different particle sizes in terms of DON. The fine-grind samples often yielded higher results than medium and coarse grinds. This trend was most pronounced in samples with higher DON content. Enzyme treatments involved either amylolytic (alpha-amylase/amyloglucosidase), proteolytic (papain) or cell wall degrading (cellulase/xylanase) enzymes. The interaction between barley sample and enzyme treatment was significant, meaning that samples did respond uniformly to the three treatments. Papain treatment resulted in significant increases (16 to 28%) in the amount of DON detected in five of the seven samples tested when compared to the untreated samples or enzyme controls. Treatment with cellulase/xylanase resulted in increased DON in three of the seven samples, while amylase/amyloglucosidase resulted in increased DON in only a single sample. The results strongly indicated that FHB infected barley samples can contain bound DON which might not be determined in the routine quantification, but can be released by proteolytic or cell wall degrading activity.