ANALYSIS OF DEOXYNIVALENOL AND DEOXYNIVALENOL-3-

GLUCOSIDE IN WHEAT

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Analysis of Deoxynivalenol and Deoxynivalenol-3-glucoside in Wheat

By

Kimberly Burgess

The Supervisory Committee certifies that this *disquisition* complies with North Dakota State University's regulations and meets the accepted standards for the degree of
MASTER OF SCIENCE

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ABSTRACT

Deoxynivalenol (DON), a mycotoxin produced in cereal grains infected by *Fusarium* Head Blight produced by *Fusarium* graminearium and Deoxynivalenol-3-β-D-glucopyranoside (DON-3G), were studied during processing using LC-MS-MS and GC.

DON reduced significantly ($P<0.05$) 61.8% during milling into flour. Therefore, DON was concentrated mostly in the bran and germ. DON increased 40.8% during the fermentation stage of baking. DON increased in dough more than flour and mixed dough.

Milling reduced by 23.7% but fermentation did not. But bread was significantly lower in DON-3G at 0.15 ppm than flour and dough at 0.31 ppm. The baking increased DON and decreased DON-3G showing a difference in stability of the mycotoxins during processing.

Enzyme hydrolysis on DON using α-amylase, cellulase, protease, and xylanase, showed a significant increase with cellulase (20.8%), protease (11.4%), and xylanase (35.6%) compared to wheat composite. DON may be bound to the cell wall or protein component of the kernel.

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I would also like to thank everyone that I have met during my time here at North Dakota State University (NDSU). You have all impacted my life for the better and hopefully I impacted yours too. I would like to give a huge thank you to my advisor Dr. Senay Simsek for all her help throughout my research. If not for her ideas, this would not have been accomplished. I also need to give a huge thank you to Kristin Whitney, research chemist/HRSW, if not for her help with the experiments and anything else that I was struggling with, I wouldn't have finished or known what to do. So thank you, thank you, thank you. I also need to give a big thank you to James Gillespie, research chemist/Barley. He conducted quite a few experiments for me and helped with the standards, so thank you again. I also want to thank my friend and former lab technician Manjusha Kasinadhuni for helping with the preliminary work and the actual baking. The next thank yous go out to Dr. Steven Qian, Pharmaceutical Sciences/LC-MS-MS and his graduate student Anna Gu for all their help in running the LC-MS-MS. I also need to thank Dr. Jae Ohm for his expertise in statistical analysis and calculating the numbers. I also need to thank Dr. Clifford Hall for agreeing to serve on my committee.

Now I would like to say thank you to my family for all of their support, love, and encouragement. I need to specifically thank my dad for suggesting and encouraging me to apply for graduate school. He also helped me find my funding, so thank you dad and I love you so much. I also need to say to my mom, thank you for all those long phone calls where I just needed to vent to someone and just the love and support you have always given me,

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INTRODUCTION

Fusarium head blight (FHB) is a major fungal disease in wheat, barley, oats, rye and maize, but less often in rice, sorghum, and triticale. It is caused by the *Fusarium* species, mainly *graminearum* and *culmorum* (Osborne and Stein 2007). *Fusarium* fungi are probably the most prevalent toxin-producing fungi of the northern temperate regions of America, Europe, and Asia (European Commission 1999). Geographical distribution of the two species appears to be related to temperature. *Fusarium graminearum* is the predominant species that causes FHB in the United States.

FHB is favored by humid or wet weather conditions during flowering and early stages of kernel development (Anonymous 2003; NDSU Extension Service 2005). FHB is recognized in the field by the premature bleaching of infected spikelet's and the production of orange, spore-bearing structures called sporodochia at the base of the glumes. During wet weather, there may be whitish, occasionally pinkish, fluffy fungal growth on infected heads in the field (Anonymous 2003).

Fusarium toxins have been shown to cause a variety of toxic effects in both experimental animals and livestock. On some occasions, toxins produced by *Fusarium* species have also been suspected of causing toxicity in humans. Deoxynivalenol (DON; vomitoxin, 12,13-epoxy-3,7,15-trihydroxy-trichothec-9-en-8-one; Figure 1) is one of the toxic secondary metabolites produced by the fungi. DON belongs to the class of trichothecenes that contain an epoxide ring (Sulyok et al 2006). DON is a very stable compound, both during storage/milling and the processing/cooking of food and does not degrade at high temperatures (European Commission 1999). The occurrence of FHB does not automatically mean that DON is present; however, a

high level of scabby kernels in the harvested grain means DON will likely be present (NDSU

Extension Service 2005).
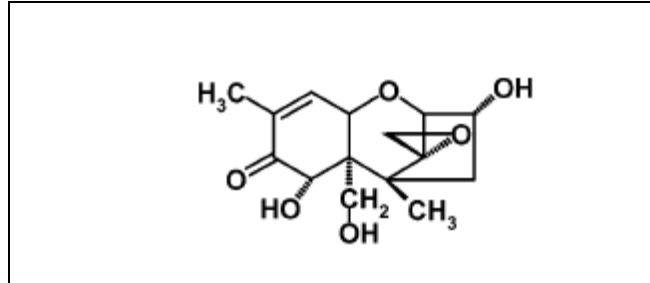
Figure 1: Deoxynivalenol structure (Berthiller et al 2005).

A direct relationship between the incidence of FHB and contamination of wheat with

DON has been established. The incidence of FHB is strongly associated with moisture at the

time of flowering (anthesis) (Osborne and Steine 2007). The timing of rainfall, rather than the

amount, is the most critical factor in *Fusarium* infection. *F. graminearum* grows optimally at

25°C and at a water activity above 0.88, while *F. culmorum* grows optimally at 21°C and at a

water activity above 0.87 (Osborne and Steine 2007).

FHB caused an estimated primary and secondary economic losses of about $10 billion

in the last 15 years for North Dakota and its surrounding wheat growing regions (North Dakota

Wheat Commission, personal communication). In addition, the presence of fungi further

deteriorates the quality of the grain and causes toxicity when consumed. In order to regulate the

human consumption of DON, the European Union legislation has set the maximum limits of 1.25

ppm for cereals, 0.75 ppm for flours and 0.50 ppm for bread (Lancova et al 2008a).

Deoxynivalenol-3-glucoside has gained considerable interest because of its universal

existence in cereals (Kushiro 2008). In response to the production of DON by fungi, DON-3G

can be formed by *Fusarium*-infected plants (Berthiller et al 2009a). DON-3G has also been

found in naturally contaminated barley as well as in malt and beer (Lancova et al 2008a). Since the fate of DON-3G after digestion is still unknown, DON-3G should be regarded as potentially hazardous to human and animal health (Sasanya et al 2008; Berthiller et al 2009a).

It is important to point out that several factors could affect the fate of a mycotoxin during food processing including the food matrix, pH, moisture content, process temperature, natural or spike contamination, and concentration of the toxin (Samar et al 2001). During food processing, heating, fermentation or other ingredients, such as enzymes, can potentially alter the mycotoxins (Berthiller et al 2009b). Bakery processing has been reported to reduce DON contamination; however, it has also been suggested that DON is highly stable during baking processes (Neira et al 1997).

OVERALL GOAL AND OBJECTIVES

1. Overall goal

The overall goal of this research was to determine the fate of both deoxynivalenol (DON) and its conjugated mycotoxin deoxynivalenol-3-glucoside (DON-3G) during milling and baking processes using gas chromatography (GC) and LC-MS-MS. The effects of various enzyme treatments on DON levels in wheat have also been investigated.

2. Hypotheses

- By removing the bran and shorts during the milling process, a decrease in DON will be seen in the final flour.
- During the baking process, the DON-3G will break down and increase the DON content in the final bread.
- Enzyme treatments will increase DON recovery of the whole wheat flour.

3. Specific objectives

- To determine the changes occurring to deoxynivalenol and deoxynivalenol-3-glucoside during milling and baking.
- To determine whether enzyme treatments have an effect on deoxynivalenol recovery in a wheat composite sample.

LITERATURE REVIEW

1. Deoxynivalenol

DON was first obtained from moldy Japanese barley, mainly infected with *Fusarium* species (Scott et al 1983). Initially, called Rd-toxin until the chemical structure was established.

Trichothecenes are tetracyclic sesquiterpenoid compounds with a 12,13-epoxy group. Group B trichothecenes are characterized with a carbonyl group at C-8 (Nagy et al 2005). Another common effect is the induction of apoptosis particulary in lymphatic and haematopoietic tissue. The DON molecule has been fully characterized as a tetracyclic sesquieterpene with seven stereo centers. In addition to two secondary and one primary alcoholic OH, two reactive functional groups are present in DON: a conjugated ketone and an epoxide. At least one but maybe both of these two functionalities may be associated with the toxic activity of DON. Conjugated ketones may undergo Michael type additions while non-symmetric epoxide rings may open readily after protonation (Nagy et al 2005). The most prominent common effects at the biochemical and cellular level are 1) strong inhibitory effect on the protein synthesis by bonding to the ribosomes, 2) inhibitory effect on RNA and DNA synthesis, and 3) toxic effects on cell membranes (Nagy et al 2005).

Trichothecenes are stable at 120°C, moderately stable at 180°C, and decompose within 30-40 minutes at 210°C (Canady et al 2001). DON is soluble in common polar organic solvents such as acetonitrile, methanol, and ethyl acetate, and is also soluble in water. The amount of toxins produced depends on the origin and the age of the fungi, as well as on the quality of the infected medium (Nagy et al 2005).

2. Conjugated mycotoxins

The first report on 'masked' mycotoxins, typically represented by a toxin bound to a more polar molecule such as glucose, amino acids, or sulphate, appeared in the mid-1980s when the severity of mycotoxicoses in animals was found to be greater than their determined levels in feed stuffs (Lancova et al 2008b). Later, Berthiller et al (2005) reported that D3G was the major form of 'masked' DON constituting up to 12% of total content of this mycotoxin in examined cereals such as wheat and maize.

Plants have the ability to metabolize mycotoxins to less toxic substances. One common method is conjugation in which mycotoxins are bound to functional groups such as sugars and amino acids (Savard 1991). Deoxynivalenol-3-β-D-glucoside (Figure 2) is a product formed by conjugation of deoxynivalenol with glucose. Poppenberger et al (2003) study showed that a gene coding for UDP glycosyltransferase enzyme in *Arabidopsis thaliana* was responsible for this conjugation of DON. These conjugated mycotoxins escape conventional analytical detection methods due to changes in molecular structures and polarities (Vendl et al 2009). Hence the name masked mycotoxins. Research has shown that masked mycotoxins are converted to their native forms in the digestive tract of humans and animals (Galaverna et al 2009). Animal exposure to DON resulted in reduction of body weight, feed refusal and adverse affects on the immune system (Sugita- Konishi et al 2006; Poppenberger et al 2003). Therefore, the actual amount of DON present in the food products is underestimated due to the presence of these masked mycotoxins.
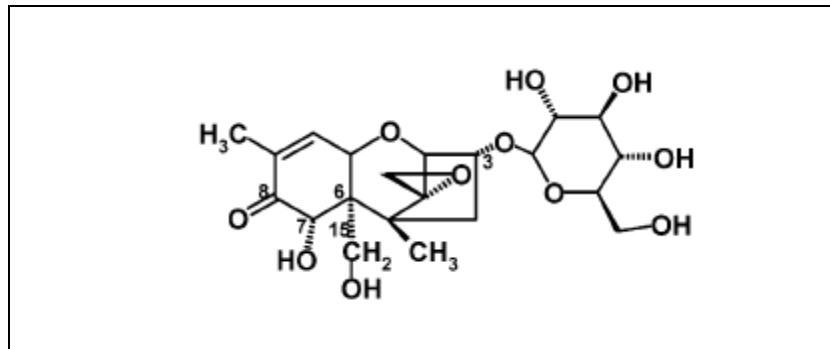
Figure 2: Deoxynivalenol-3-β-D-glucoside structure (Berthiller et al 2005).

3. Analytical method

In Europe, FHB may be caused by multiple species. In such circumstances, various species may compete with each other for resources and result in greater toxin production per unit of fungal biomass (Xu et al 2007). Since mycotoxins differ in chemical structures and properties, it is essential to use an optimized analytical method that can be used to identify a diverse range of toxins from multiple sources at the same time. Chromatographic techniques coupled with tandem mass spectrometry have evolved rapidly for analyzing mycotoxins and their metabolites (Turner et al 2009; Berthiller et al 2009a). Availability of standards for toxins makes detection and quantification possible. Berthiller et al (2005) used LC-MS-MS to identify DON-3G, along with DON, in naturally contaminated wheat samples for the first time. Researchers have used this method extensively for precise detection of a large number of toxin classes simultaneously (Vendl et al 2009; Sulyok et al 2006).

4. Effects of processing

Processing techniques can either increase or decrease the mycotoxin content that can be measured in foods (Berthiller et al 2009b). Several methods have attempted to reduce the mycotoxin content in grains. DON is distributed throughout the grains with the highest amounts being present in the bran layer. Milling the grain and removing the bran can reduce the toxin

content in the final flour (Kushiro et al 2008). Young et al (1984) also found that DON was the lowest in reduction flour, which comes from the central portion of the kernel. Dehulling grain with low levels of surface contamination was also effective in lowering the toxin content for barley, wheat and corn (Trenholm et al 1991). Abbas et al (1985) reported that cleaning was not effective in removing DON, but the concentrations were less than those found in the uncleaned wheat samples. Milling was also not effective in removing DON, but the higher concentrations were found in the outer layers on the wheat. DON was also not destroyed during bread making, but the concentrations of DON were lower in all bread samples than in the starting wheat samples. The reduction in the DON concentrations during bread making may be due to the bread-making procedure, e.g., thermal decomposition and isomerization of DON (Abbas et al 1985). Sugita-Konishi et al (2006) discovered that boiling noodles in water substantially reduced the DON content due to its water solubility.

Research determining the effect of baking on DON content has shown contradictory results. *Fusarium* infection also adversely affected the bread quality and dough properties (Dexter et al 1996). *F. graminearum* is an aggressive invader destroying starch granules, storage proteins, and cell walls and a moderate *F. graminearum* infection can cause significant compositional changes in carbohydrate, lipid, and protein (Dexter et al 1996). Lancova et al (2008a) reported no change in DON levels between flour and bread. Young et al (1984) found that DON levels increased during the baking of bread and suggested that the increase could be due to enzymatic conversion of a precursor to DON. In contrast, Samar et al (2001) and Niera et al (1997) demonstrated that DON content decreased during fermentation compared to the initial amount in different types of bread in Argentina. Although considerable research has been done

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to understand the changes in DON levels during baking, no work has been performed to determine the changes occurring to the conjugated mycotoxin D3G.

5. DON and beer

Beer contributes significantly to the diet of the population worldwide (Lancova et al 2008b). Unfortunately, barley, the major raw material used in beer production, can often be infected under field conditions by toxinogenic *Fusarium* spp., causing FHB. The barley can consequently be contaminated by mycotoxins (Lancova et al 2008b). DON is the most abundant *Fursarium* toxin in these key raw materials used for beer production.

It has been reported that while cleaning grains during a harvest or their scouring in the first stage of processing may reduce mycotoxin levels, but an increase, sometimes very distinct, of DON and other *Fursarium* toxin levels during malting. *Fusarium* toxins can be transferred from malt into beer due to their thermal stability and relative good water solubility (Kostelanska et al 2009). Research has confirmed the occurrence of detectable amounts of DON in almost all commercial beer samples, no matter where the beer was collected. According to Kostelanska et al (2009), a high dilution of fungal secondary metabolites occurs during the brewing process and the concentration in beer is typically lower by one order of magnitude compared to the concentration in raw material used for the beer production. Exposure of consumers to mycotoxins through beer should not be underestimated, particularly in the cases of high-end drinkers (Kostelanska et al 2009). Beer under certain conditions contributes significantly to intake of DON, approaching or even exceeding a tolerable daily intake (TDI) of 1 µg/kg established by the Scientific Committee on Food (SCF) in Commission Regulation 856/2005 (Kostelanska et al 2009).

It is challenging to determine the extent of transmission of mycotoxins into the final beer. The pattern of grain infection, which varies largely with technological conditions employed for the production of a particular brand of beer, must be taken into account. Kostelanka et al (2009) reported that relatively large amounts of DON-3G were present in malt and beer. The potential health risk associated with consumers' dietary exposure should be regarded as an issue of concern since DON-3G seems to be ubiquitous in various cereal-based products (Kostelanka et al 2009).

Kostelanka et al (2009) conducted a study to establish the fate of mycotoxins during the brewing process. Samples of malt, sweet wort, and final beer, obtained from several breweries, were examined. DON-dedicated ELISA kits and LC-MS-MS were used for analysis of the various brands. Kostelanka et al (2009) found that almost 74% of the samples contained DON-3G at levels exceeding the detection limit of the LC-MS-MS method, which was greater than the incidence of DON positive samples (64%) found in the same beer samples examined.

Lancova et al (2008b) reported significant increases of *Fusarium* toxins (DON, 15- and 3-acetylDON (ADONS), and HT-2 (major metabolite of T-2 toxin)) during the malting process, mainly in the germination step. In addition to these trichothecenes, the presence and formation of high levels of DON-3G was documented. Further significant increases in levels of DON-3G occurred during the brewing process. The sweet wort relative content was ten times higher than the malt grist that was taken for the processing experiment.

6. Safety issues and regulations

Many physical and chemical methods have been used to reduce mycotoxin contamination of feed stuffs, such as segregation of contaminated from non-contaminated kernels in water and saturated sodium chloride, milling, cleaning or washing, sieving and de-hulling. Baking has

been reported to reduce DON contamination; however, researchers have also suggested that DON is highly stable during the baking process. DON is currently the only regulated trichothecene in Europe according to the European Commission (EC) (Berthiller et al, 2009b). To avoid consumers' health risk due to unacceptably high dietary intake of *Fusarium* toxins, the EC has established maximum levels for DON (1.25 ppm in cereals; 0.2 ppm in processed cereal-based foods and baby foods) (European Commission 2006; Lancova et al 2008b). It does not appear that the occurrence and significance of 'masked' mycotoxins has been considered in the formulation of these limits.

Although DON is one of the least acutely toxic trichothecenes; it should be treated as an important food safety issue because it is very common in grain. In animals, moderate to low ingestion of toxin can cause a number of as yet poorly defined effects associated with reduced performance and immune function (Canady et al 2001; European Commission 2006). The main overt effect at low dietary concentrations appears to be a reduction in food consumption (anorexia), while higher doses induce vomiting (emesis). Acute symptoms of poisoning with trichothecenes are characterized by skin irritation, feed refusal, vomiting, diarrhea, hemorrhage, neural disturbance, abortion and death (Canady et al 2001; European Commission 2006). Acute effects specific to DON are nausea, vomiting, diarrhea, abdominal pain, headache, dizziness, and fever. These effects can develop within 30 minutes of exposure and are difficult to distinguish from gastrointestinal conditions attributed to microbes, such as the preformed emetic toxins from *Bacillus cereus* (Canady et al 2001; European Commission 2006). No deaths attributed to DON have been reported in humans (Canady et al 2001). DON is known to alter brain neuro-chemicals (European Commission 2006). At low doses of DON, hematological, clinical and immunological changes are also transitory and decrease as compensatory adaptation mechanisms are established

(Rotter et al, 1996). Swine are more sensitive than mice, poultry and ruminants, in part because of differences in metabolism of DON, with males being more sensitive than females (Rotter et al, 1996). Vomiting can be induced in swine and dogs with 0.05-0.1 mg/kg body weight. DON may also produce emetic effects in humans as suggested by a Chinese epidemiological study (Pestka and Smolinski 2005).

The Food and Drug Administration (FDA) currently uses advisory levels to provide guidance to the grain industry concerning levels of DON present in food or feed. These levels provide an adequate margin of safety to protect human and animal health. Currently, the FDA does not have an advisory level for DON in raw wheat intended for milling purposes; rather they rely on processors to reduce the level in finished products for human consumption to a level that does not exceed one ppm (GIPSA 2006).

7. Biochemical mode of action

At the ribosomal level, DON can inhibit the synthesis of DNA, RNA, and protein (European Commission 1999). The toxin has a hemolytic effect on erythrocytes and an acute dose of DON can induce vomiting (emesis) in pigs, whereas at lower concentrations in the diet it reduces growth and feed consumption (anorexia) (European Commission 1999) . Both effects are thought to be mediated by affecting the serotonergic activity in the central nervous system (CNS) or via peripheral actions on serotonin receptors (European Commission 1999).

The inhibition of protein synthesis following exposure to DON causes the brain to increase its uptake of the amino acid tryptophan and, in turn, its synthesis of serotonin (European Commission 1999). Increased levels of serotonin are believed to be responsible for the anorexic effects of DON and other trichothecenes. Irritation of the gastrointestinal tract may also play a role in reducing feed intake (European Commission 1999). DON has not been reported as having

carcinogenic, teratogenic or mutagenic properties (Gouze et al 2005). The general toxicity and the immunotoxicity of DON are considered to be critical effects (European Commission 1999).

8. Enzymes

Cellulase refers to a group of enzymes which, acting together; hydrolyze cellulose (Emert et al 1974; Whitaker 1971). Although cellulases are distributed throughout the biosphere, they are most manifest in fungi and microbial sources. In most familiar case of cellulase activity, the enzyme complex breaks down cellulose to beta-glucose, this is produced mainly by symbiotic bacteria in the ruminating chambers of herbivores. Alpha-amylase acts upon large linear polymers at internal bonds (Fischer and Stein 1960; Campbell and Farrell 2003). The hydrolytic products have alpha-configuration. The activity is present in all living organisms; however the enzymes vary remarkedly even from tissue to tissue within a single species (Fischer and Stein 1960). Alpha-amylase catalyzes the hydrolysis of internal alpha-1,4-glucan links in polysaccharides containing 3 or more alpha-1,4-linked D-glucose units yielding a mixture of maltose and glucose (Campbell and Farrell 2003).

Proteases are a group of protein-digesting enzymes produced by *Bacillus subtilis*, *Aspergillus oryzae*, and other microorganisms (Pommerville 2004). A protease is any enzyme that conducts proteolysis, that is, begins protein catabolism by hydrolysis of the peptide bonds that link amino acids together in the polypeptide chain forming the protein.

Xylanses belong to the pentosanases, a group of enzymes that deconstructs plant structural material by breaking down hemicelluloses, a major component of the plant cell wall (Minol et al 2011). Xylanases break down xylan, a woody, gummy, mucous substance that accompanies the cellulose present in all plants. Wheat has a high content of xylan (Minol et al 2011). Xylanses are usually used in conjunction with various other specific enzymes mainly as a

baking enzyme to improve dough qualities and to optimize the products (Minol et al 2011). Xylanases unlock the mucilaginous substances in grain to utilize them for fermentation (Minol et al 2011).

9. Preliminary DON-3G extractions

DON-3G was extracted from wheat flour according to the method described by Vendl et al 2009 (Figure 3). Usually, the conjugated derivatives of mycotoxins become more polar in nature compared to the parent mycotoxins (Berthiller et al 2009a). Solvent optimization is an important parameter especially when developing a method for determining multiple toxins simultaneously (Sulyok et al 2006). Hence, four different solvents were evaluated; neutral: acetonitrile-water (80:20 v/v), acidic: acetonitrile-water-acetic acid (79:20:1 v/v), neutral polar: acetonitrile-water (40:60 v/v), and acidic polar: acetonitrile-water-acetic acid (40:59:1 v/v). Toxins were extracted from 1 g of wheat flour using 4 ml of solvent. Extraction was performed on a shaker incubator at 180 rpm for 90 minutes. Extracts were then centrifuged at 700 rpm for 10 minutes. One ml of supernatant was transferred into a glass tube and evaporated to dryness under a stream of nitrogen. One ml of acetonitrile (10%) was added to the dried residues and filtered using 0.45 µm nylon membrane filters and stored at 4°C until further analysis. All the extractions were performed in duplicate. Among these, the acidic solvent gave the greatest quantity of DON-3G, which indicates that this solvent system can be used to extract the mycotoxins from grains for quantification.
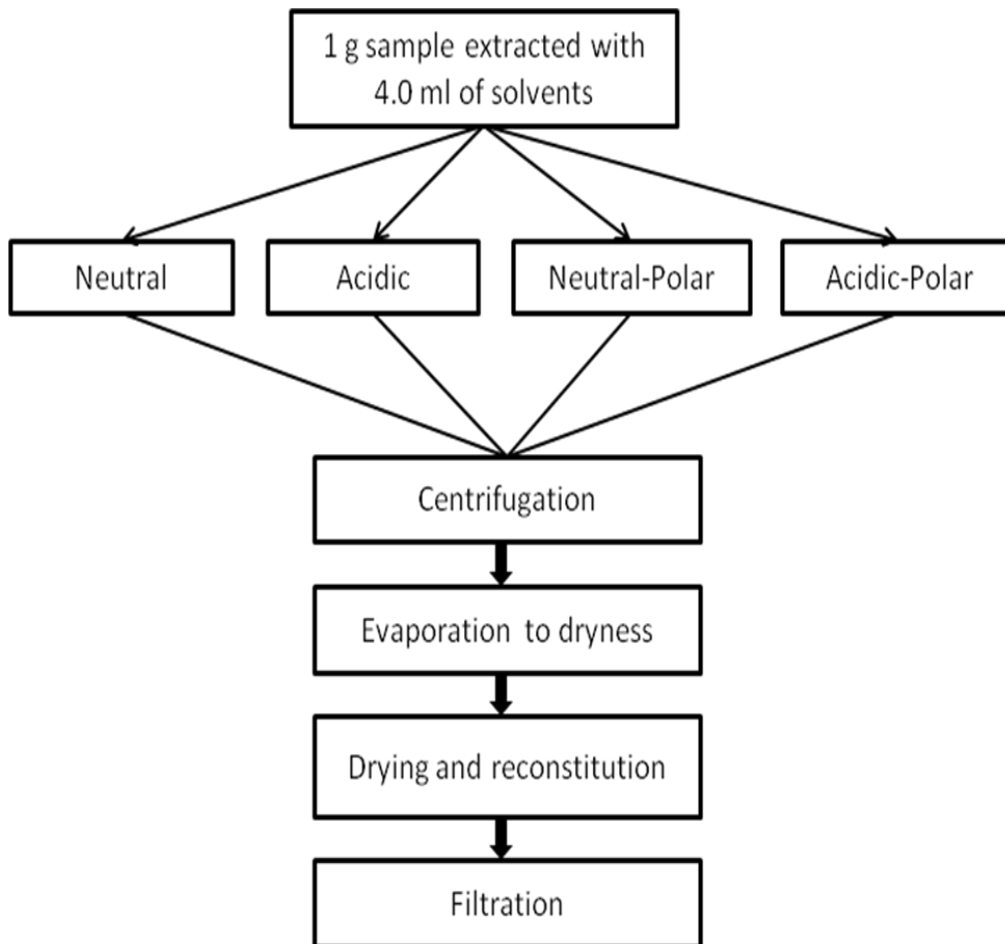
Figure 3: Deoxynivalenol-3-glucoside preliminary extraction method using four solvents.

In order to increase the concentration of DON and DON-3G, extractions were carried out using the following modifications 1) using 2 g of wheat flour during the initial step and 2) drying and reconstituting 2 ml of supernatant. These extractions were performed using neutral and acidic solvents. Neutral-Polar and Acidic-Polar solvents were not used because the extracts were difficult to filter due to the presence of other compounds. Extractions carried out using acidic solvent and 2 ml of supernatant gave the greatest quantity for both DON and DON-3G (Figure 4 and Figure 5, respectively). Figures 6 and 7 show the mass spectra of a fusarium infected wheat sample at 4.5 and 4.2 minutes, respectively.
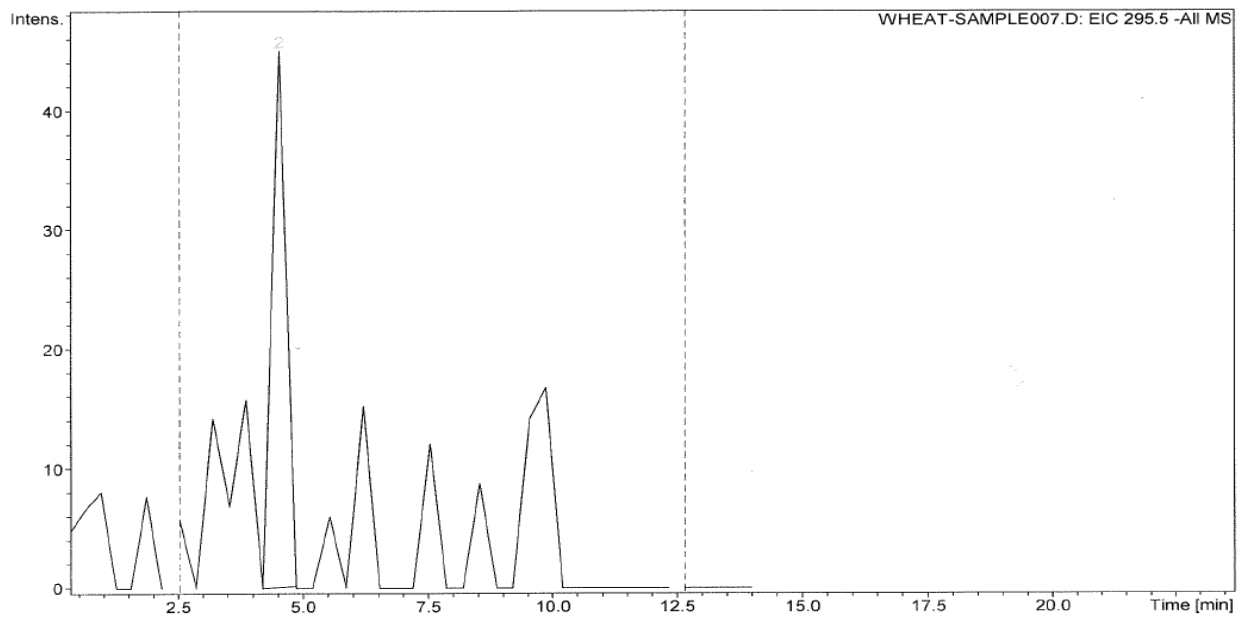
Figure 4: Ion chromatogram of deoxynivalenol in a fusarium infected wheat sample.
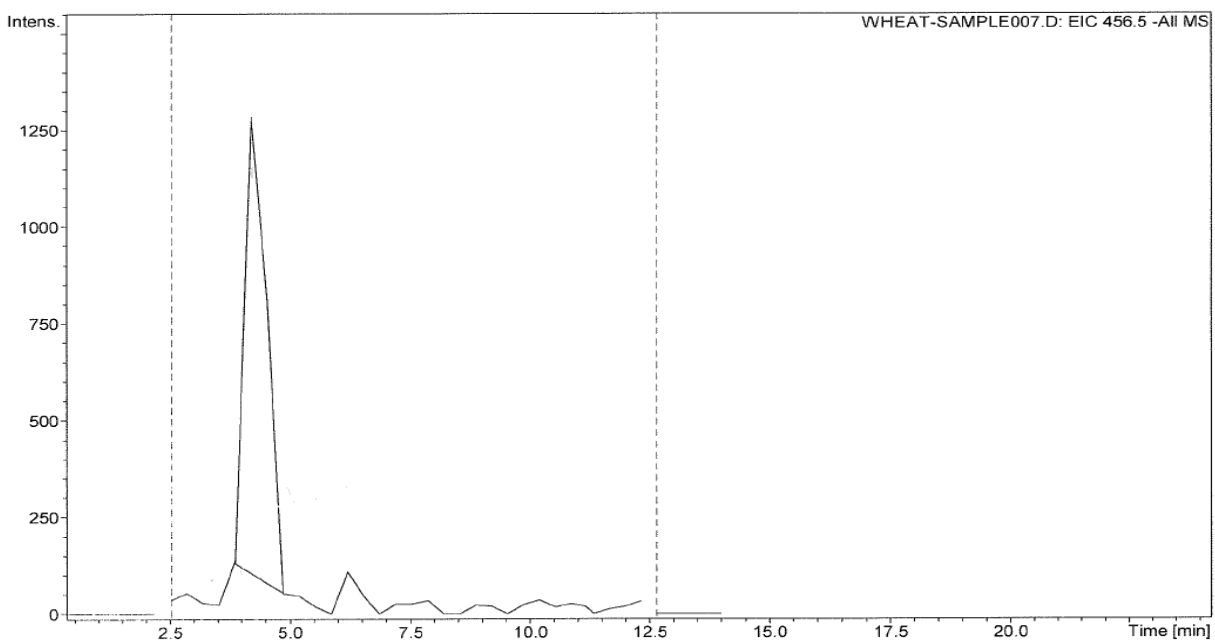
Figure 5: Ion chromatogram of deoxynivalenol-3-β-D-glucoside in a fusarium infected wheat sample.
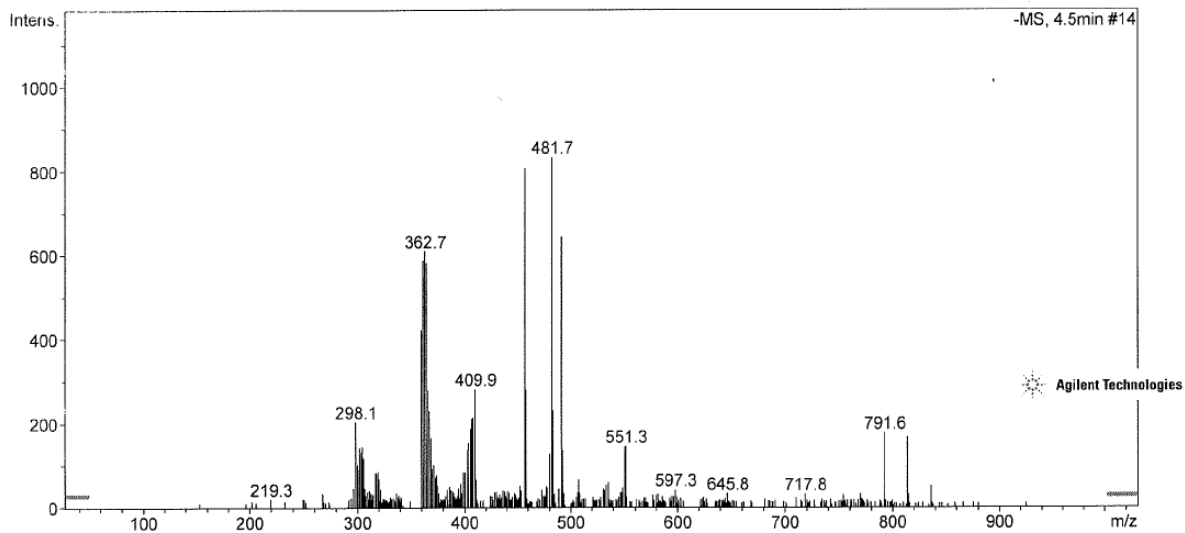
Figure 6: Mass spectrum of deoxynivalenol.
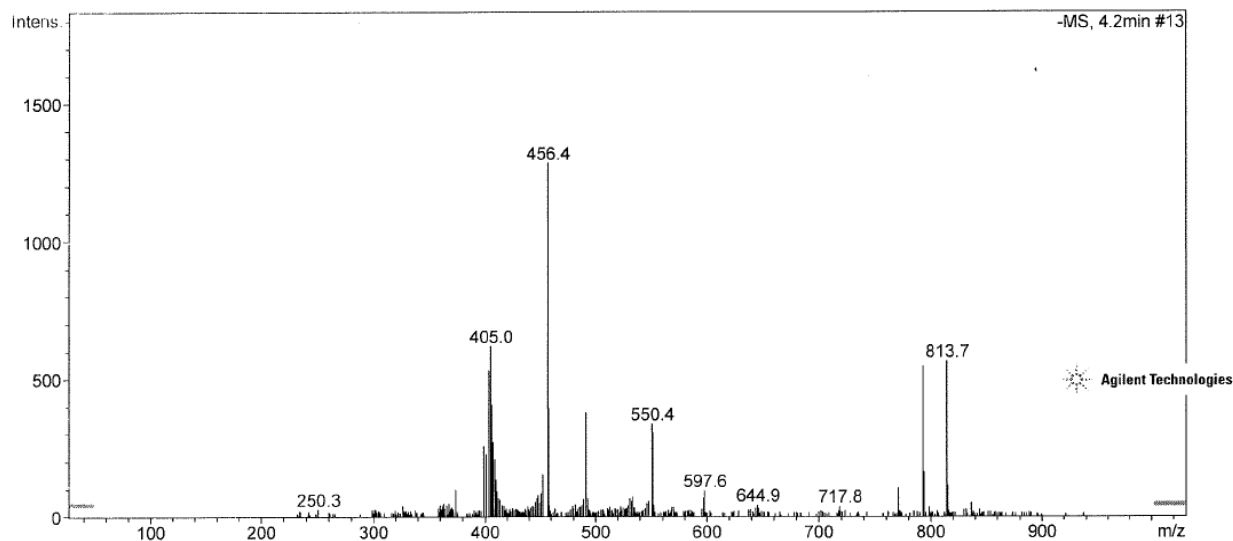
Figure 7: Mass spectrum of deoxynivalenol-3-glucoside.

From these preliminary studies, it was determined that DON and DON-3G had different

retention times of 4.5 and 4.2, thus is one run, both were detected. From these results, we

decided that DON would be detected using gas chromatography and DON-3G would use LC-

MS-MS. The preliminary studies also helped to determine that 1 g of sample was optimal for detection.

MATERIALS AND METHODS

1. Materials

1.1. Wheat samples

Hard red spring wheat variety "Freyr" grown in 2009 at the North Dakota State University Langdon Research Extension Center (Langdon, ND) was used as a raw material for this study. Wheat had previously been inoculated with *fusarium* inoculum in order to develop the disease. Freyr is moderately resistant to FHB.

1.2. DON and DON-3G standards

The DON standard used in this study was obtained from Biopure Referenzsubstanzen GmbH (Tulln, Austria). The DON-3G standard (Lot L09013A) 50.0 µg/mL in ACN (acetonitrile) was obtained from Biopure Referenzsubstanzen GmbH (Tulln, Austria).

1.3. Enzymes and enzyme activity test kits

The four enzymes used in this study were purchased from Megazyme International Ireland Ltd (Wicklow, Ireland). The four enzymes were α-amylase (Lot 100301), cellulase (Lot 40203), protease (Lot 90701) and xylanase (Lot 40903). Tablet test kits to measure α-amylase, endoprotease and xylanase (TAMZ200, PRAK, and T-XAX200) activity were obtained from Megazyme International Ireland Ltd. (Wicklow, Ireland).

1.4. Chemicals

Sodium acetate, sodium azide, 3-[N-Morpholino]propanesulfonic acid (MOPS), sodium phosphate, and sodium hydroxide (NaOH) were all purchased from Sigma. The glacial acetic

acid and hydrochloric acid (HCl) were purchased from EMD Chemicals. Calcium chloride and acetonitrile were purchased from JT Baker.

2. Methods

2.1. Moisture and protein contents

The moisture basis was determined by the approved AACC method (44-15A): Moisture-Air Oven Method. The protein content was determined by using the approved AACC method (46-30): Crude Protein-Combustion Method.

2.2. Enzyme activity

The apparent levels of α-amylase, endo-protease and xylanase were measured for the whole wheat and flour samples. The apparent α-amylase activity was determined by AACC approved method 22-05 using the amylazyme kit from Megazyme International and following the assay procedure laid out by Megazyme. Tubes with 0.5 g whole meal or flour were pre-incubated at 60°C for ten minutes with stiring. Sodium maleate (100 mM, pH 6.0, 0.74 g $CaCl_2$ and 0.02% sodium azide) buffer was pre –incubated at 60°C and 5 ml was added to the tubes and stirred for 5 minutes. The amylazyme tablets were added at 30 second intervals and the reaction was stopped after exactly 5 minutes with 2% trizma base. The slurries were then filtered and the absorbance was read at 590 nm. The apparent α-amylase activity was calculated by the following equation: Units/g = ((51.6*absorbance-0.7)/weight)*2*(1/1000), as provided by Megazyme International (AACC 1999).

Endo-protease was measured according to the method of Ichinose et al (2001), using the protazyme test tablets from Megazyme International. Extraction of protease was carried out on 0.5 g whole wheat or flour samples using sodium phosphate buffer (100 mM, pH 7.0) by stirring at room temperature for 30 minutes. The samples were then centrifuged to provide the crude

protease extract. One protazyme tablet was added to 1.0 ml of the reaction buffer (extraction buffer with 1% SDS) and stirred at 40°C for 5 minutes. The enzyme extract (1.0 ml) was added to the reaction buffer and tablet and stirred for 2 hr. The reaction was stopped with 2% trisodium phosphate (10 ml) and the samples were filtered. Finally the absorbance was read at 590 nm. One unit of enzyme activity is defined as the change in absorbance per 1 hour per 1 gram (Ichinose et al 2001).

The apparent xylanase activity of the whole wheat and flour samples was measured using a Megazyme tablet test kit according to the method set by Megazyme International with some modifications according to Courtin et al (2005). Xylanase standards were prepared from xylanase from *Aspergillus niger* that was supplied with the kit. The following dilutions of the xylanase were made, 1000, 5000, 10000, 20000 and 40000 times from the original concentration of 295 mU/ml. The flour (1.0 g) was weighed into a centrifuge tube and 5 ml sodium acetate buffer was added (25 mM, pH 4.7). The samples were shaken at 6°C and 150 rpm for 1 hour. After centrifuging (2000 rpm, 10 min.), 1 ml of the acetate buffer (for blanks), supernatant and xylanase standard dilutions were added to test tubes and incubated at 40°C for 5 minutes. Then, a xylaxyme tablet was added to each tube except for one blank tube. The samples were incubated for 17 hours at 40°C and the reaction was stopped with 5 ml of trizma base. After filteration, the absorbance was read at 630 nm and the concentrations were calculated by determining the line (slope) of the standard curve (Courtin et al 2005).

2.3. Milling

Wheat was cleaned by passing through a Carter dockage tester. The Carter dockage tester uses aspiration (air) and a combination of riddles and sieves to remove readily separable foreign material. Cleaned wheat (2 kg) was tempered to a moisture content of 16.5% prior to milling.

Wheat was milled using a MLU 202 Bühler laboratory mill. The procedure is outlined in Figure 8. The temperature in the milling room was maintained at 23-25° C and the relative humidity between 40-50%. Three milling fractions were collected: bran, shorts, and flour. Flour was prepared by mixing the break flours and the reduction flours.
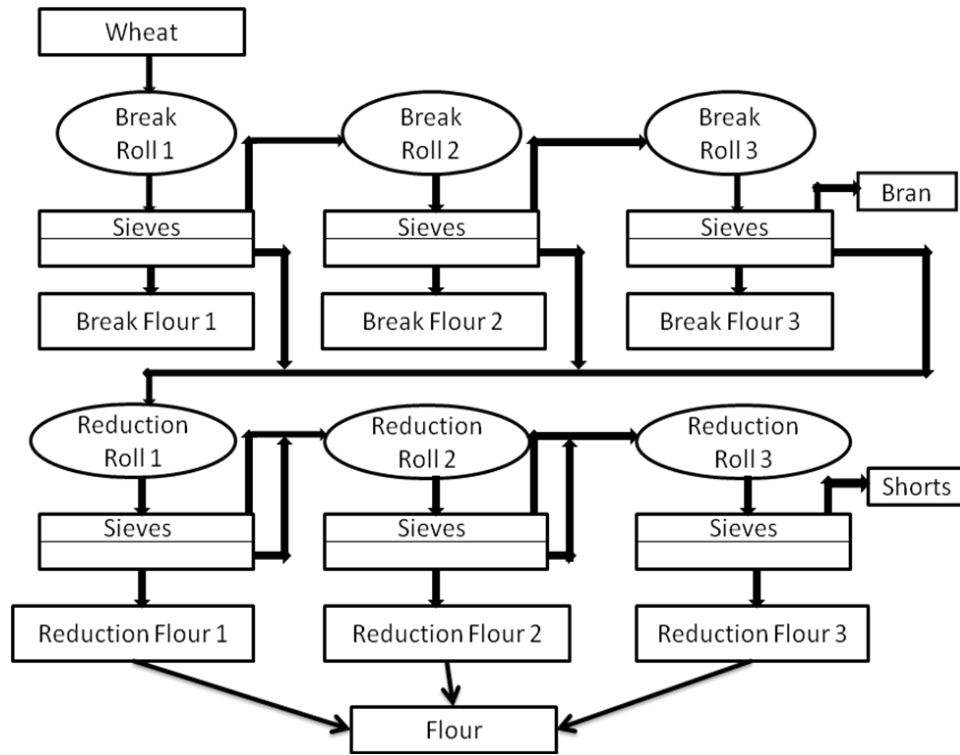
Figure 8: Flow chart of the wheat milling process.

2.4. Baking

Flour prepared by mixing the break flours and the reduction flours was used for baking. Baking was done according to the AACC straight dough method (10-09) as shown in Figure 9. Flour was measured on a 14% moisture basis. A salt (1% flour basis), sugar (5%) and ammonium phosphate (0.1%) solution was prepared in advance and maintained at 30°C. Alpha-amylase (0.1%) solution was prepared on the day of baking. Flour and yeast (1%) were placed in a mixing bowl and the liquid ingredients were added. Water was added based on absorption

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obtained from farinogram performed by Manjusha, a research assistant. Mixing was continued

until optimum dough development was determined by stretching the dough until it broke apart.

Dough was placed in a fermentation cabinet maintained at 30°C and 85% relative humidity.

Fermentation was carried out for 180 minutes followed by first dough punch after 105 minutes,

second after another 50 minutes and molding after 25 minutes. Dough was then placed in baking

pans and baked for 25 minutes at 220°C. Samples were immediately frozen followed by freeze

drying. Baking was performed over three days with replication of six samples collected per day.

The bread volume was measured using rapeseed displacement.
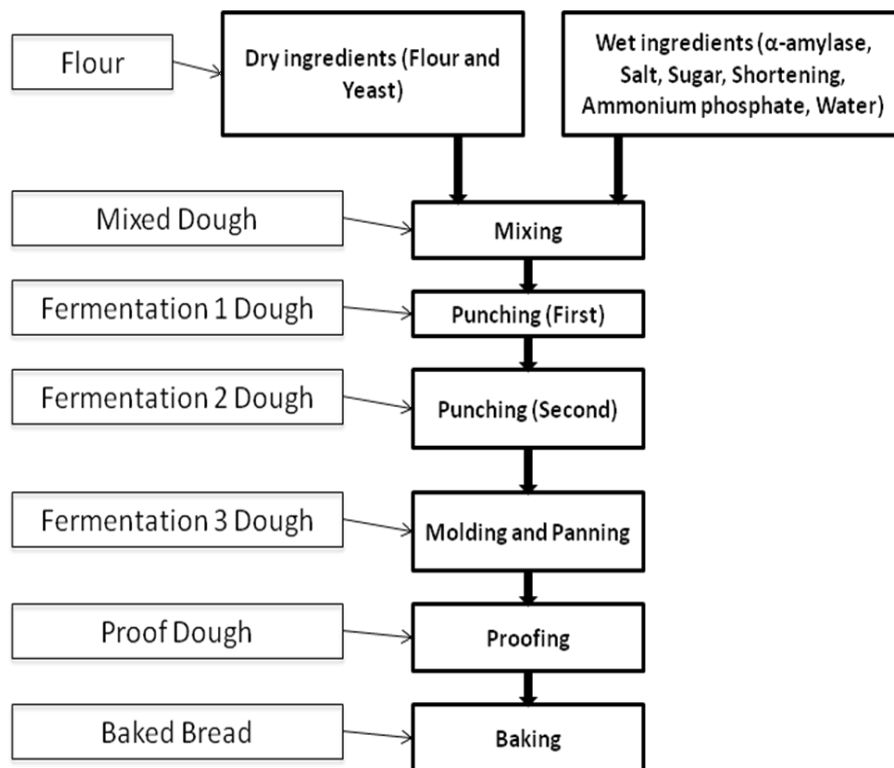
Figure 9: Standard baking method with the samples taken shown on the left side.

2.5. DON determination

Deoxynivalenol in flour samples was measured according to the method described by

Tacke and Casper (1996), with a modification of the extraction method using 2.5 g sample and

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20 mL acetonitrile-water (84%). The DON standard was prepared by dissolving 5 mg into 5 mL

acetonitrile in a 5 mL volumetric flask. The standard curves were produced by spiking clean

barley extract with a stock standard solution of DON. Gas Chromatography with Electron

Capture Detection (GC-ECD) equipment was used for DON determination. The system included

an Agilent 6890 GC with dual injector, dual cool on-column inlet, with dual DB-5 columns and

dual micro-ECD detectors (AgilentTechnologies, Inc; Wilmington, DE). This method used

helium and argon-methane (95+5) as a carrier and makeup gas, respectively.

2.6. DON-3G extraction procedure

From the preliminary experiments conducted using four solvents, it was determined that

the acidic: acetonitrile-water-acetic acid (79:20:1 v/v) solvent produced the best results (page

14). DON-3G was extracted from 1 g of wheat flour using 4 ml of solvent. Extraction was

performed on a shaker incubator at 180 rpm for 90 minutes. Extracts were then centrifuged at

700 rpm for 10 minutes. One ml of supernatant was transferred into a glass tube and evaporated

to dryness under a stream of nitrogen. One ml of acetonitrile (10%) was added to the dried

residues and filtered using 0.45 µm nylon membrane filters and stored at 4°C in glass vials until

further analysis. All the extractions were performed in duplicate.

2.7. LC-MS-MS

The LC-MS-MS system consisted of an Agilent 1200 series HPLC system

(AgilentTechnologies, Inc; Wilmington, DE) and an Agilent LC/MSD SL ion trap mass system

(Agilent Technologies, Inc; Wilmington, DE) was used for separation and detection. A gradient

chromatographic separation was performed on a ZORBAX Eclipse XDB-C18 column (Agilent,

5 µm, 75 x 4.6 mm) at 25°C. Mobile phase A consisted of 0.01% acetic acid in water, and mobile

phase B consisted of 0.01% acetic acid in acetonitrile. Drying gas flow of electrospray chamber

was 5 L/min, drying gas temperature was 225°C, and nebulizer pressure was 15 p.s.i. The

detection was made in the negative mode. For quantitative measurement, the m/z 517 (conjugate

with one acetic acid) was used for measuring of DON-3G.

2.8. Enzyme treatments for FHB wheat

2.8.1. Buffer preparation

Three buffers were prepared and used. The first buffer was 50 mM sodium acetate with a pH

of 4.7 (0.2% sodium azide) and was used for xylanase and cellulase enzyme activity. First a

beaker was filled with 850 mL deionized water and then 2.9 mL glacial acetic acid was added.

The pH was adjusted to 4.7 with 1 M NaOH, then 0.2 g sodium azide was added and the volume

was adjusted to 1 L.

The second buffer was 50 mM MOPS with a pH of 6.5 (5 mM $CaCl_2$, 0.2% sodium azide)

and was used for α-amylase enzyme activity. A beaker was filled with 400 mL deionized water

and 5.78 g MOPS was added then stirred to dissolve. Then the pH was adjusted to 6.5 with 1 M

HCL and 0.37 g $CaCl_2$ and 0.1 g sodium azide were added. Finally, the volume was adjusted to

500 mL.

The third buffer was 50 mM sodium phosphate with a pH of 7.0 (0.2% sodium azide) and

was used for protease enzyme activity. A beaker was filled with 400 mL deionized water and

2.41 g monosodium phosphate plus 2.02 g disodium phosphate were added. The pH was adjusted

with 1 M NaOH and 0.1 g sodium azide was added. Finally, the volume was adjusted to 500 mL.

2.8.2. Procedure

Whole wheat composite sample was ground with a Perten Falling Number Mill with a 0.8

mm screen. The orbital shaker was set to the appropriate temperatures of 40, 60, 45, and 50°C

for xylanase, α-amylase, cellulase, and protease, respectively. Ground wheat samples (20 g) were

weighed into three 250 ml screwcap flasks and then one of the appropriate buffers (80 mL) given

above was added. All samples were vortexed to remove all lumps. The appropriate enzyme (100

µL) was added to each flask then vortexed again to mix the solution in the flasks. The flasks

were placed into the orbital shaker (Barnstead/Lab-Line MaxQ 4000 A-Class Benchtop

Incubator, Thermo Scientific; Dubuque, IA) and shaken at 150-200 rpm for 18 hours at the

appropriate temperatures listed above. The flasks were removed from the shaker and the slurry

was poured into plastic centrifuge tubes. The tubes were frozen at -20° C. Finally, the frozen

tubes were placed in the freeze dryer (VirTis 24D x 48 General Purpose Freeze Dyer, SP

Industries, Inc; Gardiner, NY). After the samples were dried, the samples were ground using

mortar and pestle to a fine homogenous powder.

Along with each enzyme, controls were carried out using the same procedure minus the

enzyme. These controls were also performed in replicates of three. Three blanks were made of

80 ml of water and 20 g of sample and placed in a shaker at 23°C (room temperature) and shaken

at 150-200 rpm for 18 hours.

RESULTS AND DISCUSSION

1. Milling

Wheat processing most often begins with milling in order to separate the bran and germ from the endosperm to produce white flour. The milling process results in a mixing of the DON in the wheat fractions. The DON level of the flour fraction is generally lowered by about half the concentration of the whole wheat (Lancova et al 2008a). The bran and shorts milling fractions contain the remainder of the DON, which results in these fractions having a higher DON concentration than the whole wheat (Lancova et al 2008a; Nishio et al 2010). Analysis of Varience (ANOVA) was done to analyze the differences in the protein, DON and DON-3G levels of the milling fractions (Table 1). There were significant ($P<0.05$) differences between the sample means, but there were no significant differences between replicates ($P>0.05$) (Table 1).

Table 1: ANOVA results for protein, DON and DON-3G contents of samples collected from wheat milling.

Variable	Source	df	Sum of squares	Mean square	F Value	Pr > F
Protein						
	Treatment	3	27.49	9.165	636.3	<.0001
	Replicate	1	0.0000007	0.0000007	≤ 0	0.9946
	Error	11	0.101	0.014		
DON						
	Treatment	3	404.816	134.939	85.34	<.0001
	Replicate	1	0.005	0.005	≤ 0	0.9567
	Error	11	11.068	1.581		
DON-3G						
	Treatment	3	0.199	0.066	17.71	0.0012
	Replicate	1	0.016	0.016	4.32	0.0762
	Error	11	0.026	0.004		

As found in a previous study from Abbas et al (1985), the milling process significantly ($P<0.05$) lowered the DON level in the flour about 1.6 to 9.9%. However, milling was not effective in removing DON completely. Young et al (1984) found that the mycotoxin could be

produced at the site of fungal growth rather than transported from the kernel surface to the interior. In our wheat sample there was 61.8% lower concentration of DON in the flour (Table 2) compared to the whole wheat. There was a significantly higher concentration of DON in the bran and shorts milling fractions, and was nearly triple that of the whole wheat (Table 2). The high DON concentration in the bran and shorts fractions is a result of the *F. graminearium* fungus infecting from the outside of the wheat kernel (Abbas et al, 1996; Nishio et al, 2010; Hazel and Patel 2004). Hazel and Patel (2004) stated that typical results show the flour has about half the DON level of the cleaned wheat, while the bran can have levels two or more times greater than the wheat. The DON levels detected in the bran and shorts milling fractions were higher compared to the whole wheat. This is about three times more than the DON concentration detected in the whole wheat. However, the DON concentration was significantly lower between the whole wheat and flour samples. This trend is supported by other studies that stated that the highest DON content is located in the bran and shorts milling fractions (Lancova et al 2008a; Kushiro 2008; Abbas et al 1985).

There are also some significant differences between the DON-3G levels in the milling fractions. There was a 23.7% decrease in DON-3G concentration between the whole wheat and the flour but it was not found to be significant (P>0.05) (Table 2). The DON-3G levels detected in the bran and shorts fractions were significantly higher than both the whole wheat and the flour (Table 2). DON-3G in the bran and shorts shows the same increase detected, but the higher levels are not as high as the DON levels detected. The bran and shorts have about two times the DON-3G concentration detected as the whole wheat. The bran and shorts have a higher DON-3G concentration, because the mycotoxins were produced around the bran and shorts and cannot be transported from the surface into the interior (Young et al 1984). However, the difference in

DON-3G content detected from whole wheat to flour was not significant because the DON-3G

also cannot be completely removed by milling.

Table 2: Protein and mycotoxin content of four wheat samples collected during milling process.

	Protein	DON‡	DON-3G §
	%†	ppm*	ppm
Whole wheat	13.2c	5.23b	0.38b
Bran	16.0b	14.30a	0.59a
Shorts	16.3a	15.63a	0.58a
Flour	13.0c	2.00c	0.29b
LSD (0.05)	0.2	2.43	0.12

†14% moisture basis;
‡ DON: Deoxynivalenol;
§DON-3G: Deoxynivalenol-3-glucoside
*ppm: parts per million; Values in the same column with the same letter are not significantly different ($P>0.05$).

2. Baking

The baking process followed the AACC straight dough method (10-09). The final flour was created by combining the break and reduction flours. ANOVA was done to analyze the differences in the protein, DON and DON-3G levels of the baking process samples taken at mixing, punching 1, punching 2, molding and panning, proofing, and final baked bread (Table 3). There were significant differences between the sample means ($P<0.05$), but there were no significant differences between replicates ($P>0.05$) (Table 3). Protein was analyzed in order to determine whether there was a dilution effect on DON due to the extra ingredients added during the preparation of the mixed dough. A lean formula was used to produce the bread in order to minimize the dilution of the flour in the bread.

Table 3: ANOVA results for protein, DON and DON-3G in wheat subjected to the bread making process.

Variable	Source	df	Sum of squares	Mean square	F Value	Pr > F
Protein						
	Treatment	5	1.267	0.253	47.68	<.0001
	Replicate	1	0.001	0.001	0.11	0.742
	Error	11	0.0584	0.0053		
DON						
	Treatment	5	7.031	1.406	10.69	0.001
	Replicate	1	0.653	0.653	4.97	0.048
	Error	11	1.447	0.132		
DON-3G						
	Treatment	5	0.047	0.009	9.28	0.001
	Replicate	1	0.0002	0.0002	0.21	0.653
	Error	11	0.011	0.001		

In previous studies focusing on the changes of DON during the baking process, Samar et al (2001) observed a 41% reduction in DON for French bread and a 25% reduction for Vienna bread. Pacin et al (2010) reported a mean reduction of DON between flour and products for French bread at 42.3% and for Vienna bread at 58.3% reduction, respectively. Abbas et al (1985) and Neira et al (1997) reported 16.8% and 96.6% minimum and maximum reduction rates, respectively, between the doughs and final products. Kushiro (2008) and Young et al (1984) detected lower DON concentration from bread samples compared to the wheat flours used for preparation. Each of these studies used a different method for producing the bread samples. Some researchers de-fatted the samples prior to the acetonitrile:water extraction, while others focused on different fermentation times. Lancova et al (2008a) observed higher DON levels of 21-40% in the kneaded dough, but saw a significant decrease during fermentation of approximately 38-46% of the original content. However, Lancova et al (2008a) also saw relatively high DON levels after the proofing stage ranging from 132-145%, but when comparing the DON content between the flours and the final products, practically no changes occurred. In

this study, there was an increase in DON concentration detected from the mixed dough sample to the final baked bread of 40.8% (Table 4). There was an increase of 1.06 ppm between the mixed dough and the first fermentation step. After proofing, the dough had 1.96 ppm higher DON than the mixed dough and the baked bread had 1.36 ppm higher DON than the mixed dough. Although there was a slight decrease in DON detected after baking compared to the proofed dough, it was not significant ($P>0.05$).

There was a trend of increasing DON through the fermentation and proofing stages with a slight but not significant decrease from proofing to baked bread. However, there was still a higher level of DON detected than what was found in the mixed dough. The trend contradicts other studies (Samar et al 2001, Abbas et al 1985, Neira et al 1997, Pacin et al 2010, Kushiro 2008, and Young et al 1984) that stated there was a reduction in DON detected during fermentation. There could be a contradiction between this research and the research mentioned above due to different samples used and different materials and methods being used and followed.

There was a significant difference between the DON-3G levels of the dough samples and the baked bread. The baked bread had 51.6% less DON-3G detected compared to the mixed dough ($P<0.05$) (Table 4). The DON-3G levels detected in the dough during fermentation and proofing were not found to be significantly different from the mixed dough sample ($P<0.05$) (Table 4). A significant reduction of 42.3% in DON-3G was observed between the proofing and baking steps. The DON-3G content was stable until the baked bread, which had significantly lower DON-3G than the other samples. The trend for DON--3G during the fermentation and proofing stages shows that DON-3G may not have an impact on the DON level, which showed an increase in the DON detected during the same stages. However, the final baked bread showed

a decrease in both DON and DON-3G, this could imply that neither are as heat stable as

previously thought.

Table 4: Protein and mycotoxin contents of wheat samples subjected to the bread baking process.

	Protein %†	DON‡ ppm*	DON-3G§ ppm*
Mixed dough	12.4e	1.97c	0.31a
Fermentation 1 dough	12.6d	3.03b	0.29a
Fermentation 2 dough	12.8c	3.43ab	0.29a
Fermentation 3 dough	12.8c	3.63ab	0.26a
Proof dough	13.0b	3.93a	0.26a
Baked bread	13.2a	3.33ab	0.15b
LSD (0.05)	0.1	0.65	0.06

†14% moisture basis;
‡DON: Deoxynivalenol;
§DON-3G: Deoxynivalenol-3-glucoside
*ppm: parts per million; Values in the same column with the same letter are not significantly different ($P>0.05$).

Other possible mechanisms or causes for reduction of DON levels might involve the

release of DON from its conjugated form and probably followed by its transformation into a

substance that cannot be determined by conventional analysis (Lancova et al 2008a).

Unfortunalty its nature remains unclear. It also has not been proved that reduction in DON

content was due to its binding to yeast cells (Lancova et al 2008a). Pronounced changes of DON

levels in dough samples taken in particular steps of baking process might be due to

transformation of DON forms (free/conjugate). More research is needed to explain these

phenomena (Lancova et al 2008a).

3. Enzyme treatment

To find out if enzymes commonly found within wheat and wheat processing had any

affect on the DON content, four common enzymes, α-amylase (starch hydrolysis), cellulase

(cellulose hydrolysis), protease (protein hydrolysis), and xylanase (xylan hydrolysis), were

chosen. These enzymes hydrolyze the major components of the wheat to which the DON may be

bound. Specifically the cellulase and xylanase were used to hydrolyze cell wall material.

Protease was used to hydrolyze the protein component of the bran, germ and gluten proteins and

α-amylase, to degrade the starch in the endosperm.

An ANOVA analysis was conducted on the enzyme treated wheat (Table 5). There were

significant differences between the sample means ($P<0.05$), but there were no significant

differences between replicates ($P>0.05$) (Table 5).

Table 5: ANOVA results based on data from enzyme treated wheat samples.

Source	df	Sum of square	Mean square	F Value	Pr > F
Treatment	8	37.39	4.674	30.24	<.0001
Replicate	2	0.148	0.074	0.48	0.629
Error	8	2.473	0.155		

Table 6 shows the differences detected in the DON content between the four enzyme

treatments and their buffer controls. There were no significant differences detected in DON

between α-amylase and the MOPS buffer from the wheat composite ($P<0.05$). Cellulase treated

sample 20.8% higher DON concentration than the wheat composite extracted by sodium actetate.

However, the sodium acetate buffer control for cellulase showed a 23.3% increase in the DON

levels detected. The protease and xylanase showed significant changes between the enzymes and

the buffers used for their controls. The sodium phosphate buffer used for the protease control

showed a 26.3% increase in DON detected, where the protease enzyme only had an 11.4%

increase in the DON levels detected. The highest level in DON detected occurred with the

xylanase enzyme (35.6%) and its sodium acetate buffer (26.9%) ($P<0.05$).

Table 6: DON content found in enzyme treated wheat samples on a dry weight basis.

Treatment	pH	Temperature °C	DON† ppm*
Wheat Composite	--	--	5.70d
MOPS	6.5	60	5.45d
α-amylase	6.5	60	5.17d
Sodium Acetate‡	4.7	45	7.43b
Cellulase	4.7	45	7.20b
Sodium Phosphate	7.0	50	7.73b
Protease	7.0	50	6.43c
Sodium Acetate§	4.7	40	7.80b
Xylanase	4.7	40	8.85a
LSD (0.05)			0.68

†DON: Deoxynivalenol
‡Sodium acetate buffer control used for cellulase treatment;
§Sodium acetate buffer control used for xylanase activity treatment
*ppm: parts per million; Values in the same column with the same letter are not significantly different ($P>0.05$).

The difference in DON levels recovered between the α-amylase enzyme and the other three enzymes could be that α-amylase hydrolyzes the starch to dextrins, maltose, and glucose to provide fermentable sugars for yeasts. DON may not be bound to the polysaccharides of starch, which makes up the endosperm or interior of the kernel, which may not be highly contaminated by the *F. graminearium* fungus. The outerlayers of the wheat kernel receives the majority of the fungus exposure and the other enzymes which hydrolyze components found in the wheat bran and germ may release the bound DON found in these areas. From the samples that were treated with the four enzymes, the next progression in the study was to see where the four enzymes fell within a typical range by using enzyme activity kits.

According to Nightingale et al (1999), proteolytic enzymes capable of digesting durum and bread wheat storage proteins are found in *Fusarium* damaged kernels by both *F. graminearum* and *F. avenaceum*. The lower concentration of endosperm protein in *Fusarium*

damaged kernels is probably due to enzymatic digestion of protein sometime after deposition (Nightingale et al 1999). Proteolytic enzymes associated with *Fusarium* damage in bread wheat will hydrolyze endosperm proteins during dough mixing and fermentation, resulting in weaker dough and decreased loaf volume. This decrease in dough functionality and loaf volume will be accentuated in baking processes that involve long fermentation periods (Nightingale et al 1999).

There were only two buffers used, sodium acetate and sodium phosphate, that worked better alone than with the enzymes. These two buffers could have reacted with the already present cell-wall degrading enzymes that were produced by the fungus in order to invade the plant cell (Wang et al 2005). The buffers allowed present enzymes to function in the appropriate pH range. Thus, the buffers are not really anything but buffering the pH; however, with the correct pH range, the enzymes available are able to degrade the plant cell causing the possibility of DON levels to be increased.

4. Enzyme activity

Knowledge of basic enzyme kinetic theory is important in enzyme analysis in order both to understand the basic enzymatic mechanism and to select a method for enzyme analysis (Holum 1968; Martinek 1969). The conditions selected to measure the activity of an enzyme would not be the same as those selected to measure the concentration of its substrate (Holum 1968; Martinek 1969). Several factors affect the rate at which enzymatic reactions precede - temperature, pH, enzyme concentration, substrate concentration, and the presence of any inhibitors or activators (Holum 1968; Martinek 1969).

From the results of the enzyme treatment experiment where a possible increase in DON was seen, it was decided to investigate the enzyme activity of the samples used in this research. The amylase activity in FHB infected wheat sample used in this study falls within the middle to

upper end of the typical range for sound wheat (Table 7). The protease activity in the infected

wheat sample was higher than the typical range for sound wheat and xylanase activity in the

infected wheat sample was at the upper end of the typical range for sound wheat. The high

activity of xylanase could be causing the DON levels to be increase, due to the breakdown of cell

wall material (Table 7). The enzymes in the wheat, such as xylanase, could be releasing DON

during any step throughout the baking process. This could be caused by the changes in pH,

becoming lower, or the increase in temperature causing the enzymes to breakdown the material

faster.

Table 7: Enzyme activity from wheat samples measured using tablet test kits.

	Amylase* Units/g	Protease* A590/hr/g	Xylanase* mU/g
Typical ranges for enzyme activity in wheat			
Whole wheat	0.05-0.2	1.2-1.9	0.5-29.5
Flour			0.1-21.5
Samples			
Whole wheat†	0.103	2.70	26.53
Flour‡	0.074	1.88	12.51
Wheat composite§	0.151	2.80	28.18

*Data taken from a set of previously analyzed sound samples
**Data taken from several sets of previously analyzed sound samples
†Milling and baking samples
‡Milling and baking samples
§Enzyme treated samples

CONCLUSIONS

The goal of this research was to determine the fate of both deoxynivalenol and deoxyivalenol-3-glucoside during milling and baking processes using gas chromatography (GC) and HPLC-MS-MS, respectively. Four enzymes were also used to treat the samples to investigate what happens with the deoxynivalenol content when the plant matrix is broken down. To accomplish these goals, three hypotheses were deteremined. The first hypothesis stated that by removing the bran and shorts during milling, a decrease in DON would be seen in the final flour. The second hypothesis stated that during the baking process DON-3G would breakdown and increase the DON levels in the final bread and finally, the third hypothesis stated that enzyme treatments would increase the DON content of the whole wheat flour.

This research supported the first hypothesis, that the DON decreased in the final flour after the bran and shorts were removed. The second hypothesis turned out to be open ended with the results. The DON levels did show some increase throughout the baking process, especially the fermentation and proofing stages, but with a slight decrease after the final baked bread; whereas, DON-3G did not show any significant change throughout fermentation and proofing, but did decrease in the final baked bread. Further research needs to be performed before this hypothesis can be supported. The third hypothesis needs further study. Of the four enzymes chosen, xylanase and cellulase enzymes along with the buffer sodium acetate had the highest increase of DON detected. This increase in DON could be due to the fact that both xylanase and cellulase degrade plant wall material where some of the DON is bound. This could also release any DON bound within the fungus causing the whole wheat flour increasing DON detection levels.

FUTURE RESEARCH

The next steps in this research would be to focus more on the enzyme treatments and their activity. Conditions would need to be improved in order to detect the DON-3G using the enzyme treatments. Another idea would be to study the enzyme activity throughout the baking process, from mixing to the final baked bread. Do any factors from the baking process have an impact on the four enzymes used for treatment?

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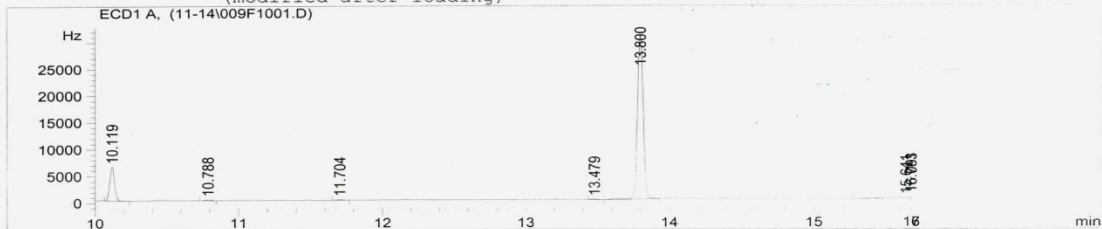dearalenone. Journal of Agricultural and Food Chemistry 39:356-360.

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APPENDIX

Batch Run # 7 of 41
Data File C:\HPCHEM\1\DATA\11-14\009F1001.D   Sample Name: wt pool

==
    Injection Date : 5/12/2011 3:49:35 AM   Seq. Line :   10
    Sample Name   : wt pool   Location : Vial 9
    Acq. Operator   : Kelly Benson   Inj :   1
    Acq. Instrument : Instrument 1   Inj Volume : 1 µl
    Acq. Method   : C:\HPCHEM\1\METHODS\RUNGC.M
    Last changed   : 5/12/2011 2:12:35 AM by Kelly Benson
    Analysis Method : C:\HPCHEM\1\METHODS\DONF.M
    Last changed   : 5/12/2011 8:39:20 PM by Kelly Benson
                      (modified after loading)

```
ECD1 A, (11-14\009F1001.D)
Hz
                                                         13.800
25000
20000
15000
           10.119
10000
 5000           10.788       11.704         13.479            15.665
    0
      10        11         12         13         14        15        16      min
```

==
                     Internal Standard Report
==

Sorted By   :   Signal
Calib. Data Modified :   Thursday, May 12, 2011 8:39:14 PM
Multiplier   :   4.0000
Dilution   :   1.0000
Use Multiplier & Dilution Factor with ISTDs
Sample ISTD Information:
ISTD ISTD Amount   Name
  #   [ppm]
----|-------------|--------------------------
  1   5.00000e-1   Mirex

Signal 1: ECD1 A,

RetTime Type   Area   Amt/Area   Amount   Grp   Name
 [min]   [Hz*s]   ratio   [ppm]
-------|------|----------|-----------|----------|--|------------------
 10.119 BB S   1.43873e4   2.92221 9.71955e-1   DON
 11.476   -   -   -   3-ADON
 11.704 PBA   303.62405   4.76789 3.34671e-2   15-ADON
 12.008   -   -   -   NIV
 13.800 BB S I 8.65117e4   1.00000   2.00000   Mirex

Totals without ISTD(s) :   1.00542

 Results obtained with enhanced integrator!
2 Warnings or Errors :

Warning : Calibration warnings (see calibration table listing)
Warning : Calibrated compound(s) not found

==
                     *** End of Report ***

Instrument 1 5/12/2011 8:39:57 PM Kelly Benson   Page 1 of 1

Figure A1: Deoxynivalenol chromatogram from gas chromatography with electron capture detection.
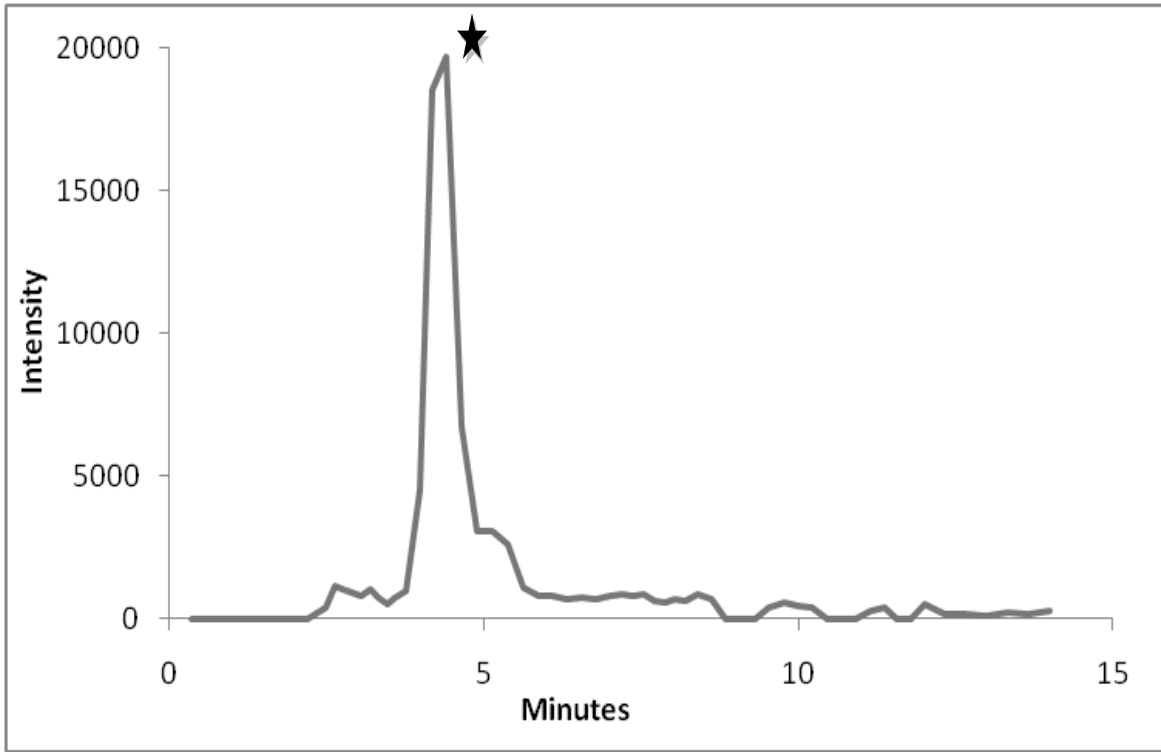
Figure A2: Deoxynivalenol-3-glucoside standard 6.0 ppm from HPLC chromatogram.