OCHRATOXIN A AND OCHRATOXIGENIC FUNGI IN

FRESHLY HARVESTED AND STORED BARLEY AND WHEAT

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ABSTRACT

Ochratoxin A (OTA) is a toxin produced both prior to harvest and during storage by *Penicillium* and *Aspergillus* species in a variety of commodities. Although several studies have been conducted in Europe and Canada examining the occurrence and concentration of OTA in cereal grains, data is lacking for the United States, where guidance levels and regulations do not exist. This study aims to fill in the knowledge gaps surrounding OTA and ochratoxigenic fungi in barley and durum and hard red spring wheat grown in the northwestern and Upper Great Plains regions of the United States. In total 2.7% (n = 37) of the 1370 samples taken over 2 consecutive years had detectable levels of OTA (0.15-9.11 ng/g) directly after harvest. The number of positive samples was significantly greater in 2012 compared to 2011. This difference may be due to weather conditions during the planting and growing seasons or simply natural variation between years. Stored barley and wheat (N = 262) had a higher prevalence (12.2%) and greater range (0.16-185.24 ng/g) of OTA compard to freshly harvested samples. Although 81.3% of the OTA-positive samples had been stored for ≥6 months, samples that had been stored for as short as 1 month also tested positive. These results underline the importance of proper storage conditions in minimizing OTA contamination. *P. verrucosum* was found to be the primary ochratoxigenic species in these samples. Of the 110 isolates tested, 64.7% were confirmed OTA producers. Samples containing >1 ng/g OTA had significantly more OTA-producing *P. verrucosum* strains than samples with undetectable OTA. Infestation rate did not correlate with OTA level. Additionally, OTA concentration did not correlate with *otanps*PN, an OTA biosynthesis gene. This indicates that the concentration of *P. verrucosum* in a sample may increase the likelihood of contamination but is not a reliable indicator of OTA level.

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LIST OF ABBREVIATIONS

ANOVA .... Analysis of Variance

AOAC .... Association of Analytical Communities

a_w .... Water Activity

AFLP .... Amplified Fragment Length Polymorphism

BEN.. Balkan Endemic Nephropathy

CCA .... Coconut Cream Agar

CFU.. Colony Forming Unit

CGC .... Canadian Grain Inspection Commission

Ct .... Threshold Cycle

CV ....Coefficient of Variance

CYA .... Czapek Yeast Agar

CZE-LIF....................................Capillary Zone Electrophoresis with Laser Induced Fluorescence

DG18.. Dichloran 18% Glycerol

DNA.. Deoxyribonucleic Acid

DON.. Deoxynivalenol

DRBC... Dichloran Rose Bengal Chloramphenicol

DRYES .... Dichloran Rose Bengal Yeast Extract Sucrose

DYSG.. Dichloran Yeast Extract 18% Glycerol

EFSA... European Food Safety Authority

ELISA .... Enzyme-Linked Immunosorbent Assay

ESI .... Electrospray Ionization

EU .... European Union

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CHAPTER 1. INTRODUCTION

Ochratoxin A: A Brief History

When the mysterious turkey X disease in England was linked to peanut meal contaminated with secondary metabolites from *Aspergillus flavus* in 1960, it brought to scientists' attention the possibility that other unknown fungal metabolites might be equally deadly. This prompted large-scale screenings, a period between 1960 and 1975 which has been dubbed the "mycotoxin gold rush", targeting mycotoxin discovery and identification (*22*). In 1965 ochratoxin A (OTA) was discovered as a result of intentionally inoculating *Aspergillus ochraceus* into corn meal (*53, 194*). Four years later naturally occurring OTA was isolated for the first time from a commercial corn sample (*181*). Although hundreds of mycotoxins have been identified to date, OTA remains one of roughly twenty mycotoxins known to occur in foodstuffs frequently enough and at sufficient levels to cause food safety concerns (*45*).

The first evidence that exposure to OTA had serious health implications occurred in the 1980s, during which time OTA was believed to be responsible for an endemic porcine nephropathy in Denmark, Sweden, and Poland and was implicated in endemic human nephropathies described in the Balkan region (*84, 94, 95*). More recently OTA has been found to be the cause of chronic interstitial nephropathy in North African countries (*86, 169*). The deleterious effects of OTA have also been described in other parts of the world but studies to determine large-scale population exposure to OTA have yet to be carried out (*53*). In 1993, general principles of European Union (EU) legislation on contaminants in food were established and in 2001 maximum limits for ochratoxin A (as well as aflatoxins and patulin) in food were set (*47, 63*). Other countries followed suit and today over 99 countries regulate OTA at various

levels in commodities and food (*97, 195,199*). Currently, the United States does not regulate OTA in any commodity or food.

Literature Review

Mycotoxins

Mycotoxins are secondary metabolites produced largely by the genera *Aspergillus*, *Fusarium*, and *Penicillium* although other genera are known to produce mycotoxins including *Byssochlamys*, *Neosartorya*, and *Eupenicillium* (*31*). *Fusarium* species are plant pathogens whereas *Aspergillus* and *Penicillium* species infect cereal grain during storage (*56*). To date over 300 mycotoxins, representing a wide range of structures and toxic effects have been described (*56, 127*). Although the ecological reasons by which secondary metabolites are produced have yet to be completely elucidated it is widely accepted that their role is multifunctional, one of which is aiding in survival (*173*). In most cases humans and animals are exposed to mycotoxins as a result of ingestion or inhalation (*56, 210*).

Ochratoxins

The ochratoxins are a group of pentaketides, made up of a dihydroisocoumarin linked to L-β-phenylalanine by an amide bond (*18*). OTA is composed of phenylalanine linked to a chlorinated dihydroisocoumarin ring via an amide bond (*77*). Several analogues of OTA exist. These include ochratoxin B (OTB), ochratoxin C (OTC), ochratoxin α, and ochratoxin β. OTB differs from OTA in that it is dechlorinated. OTC has an ethyl ester on the carboxylic group of the phenylalanine moiety (Figure 1) (*18*). Ochratoxin α lacks the phenylalanine moiety (*210*). Ochratoxin β is an intermediate in the OTA biosynthetic pathway and is the dechlorinated form of ochratoxin α (Figure 2) (*3*).

Figure 1.1. Chemical structure of ochratoxins A, B, and C.

Ochratoxin A
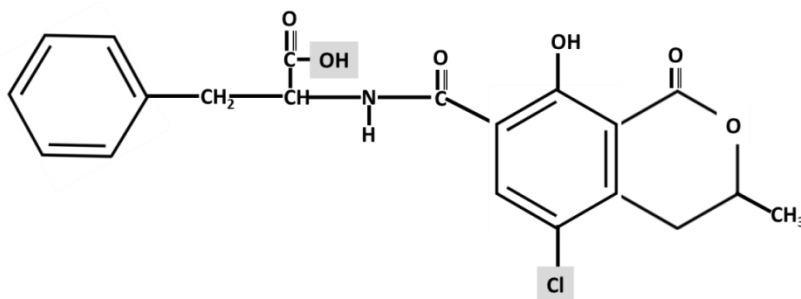
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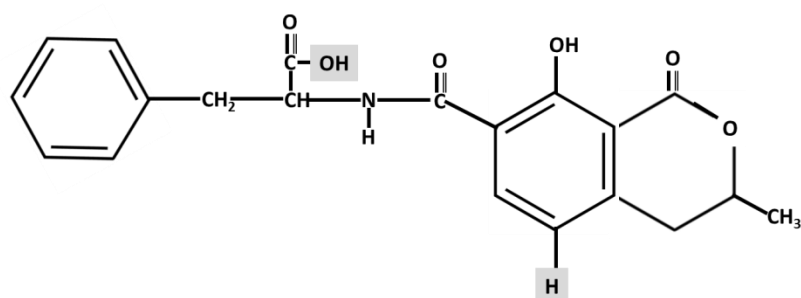
Ochratoxin C
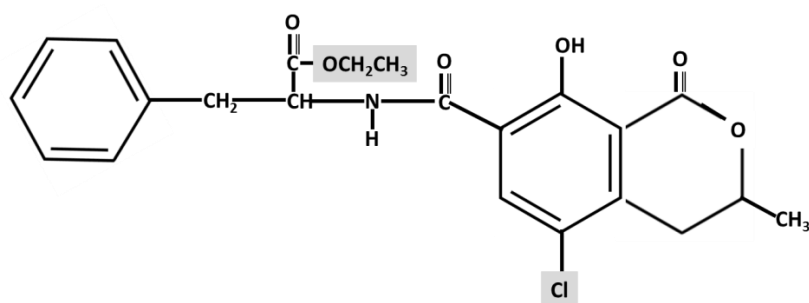
Adapted from Yu, 2011.

Figure 1.2. Chemical structure of ochratoxins α and β.
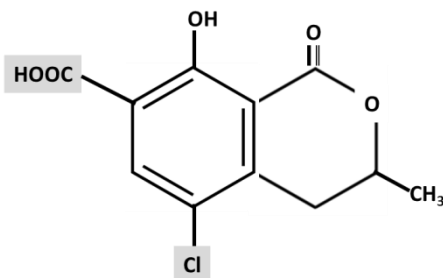
Ochratoxin α
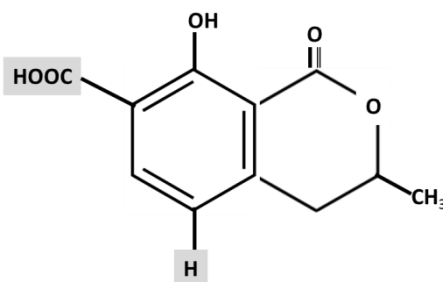
Ochratoxin β

Adapted from Harris and Mantle, 2001.

Ochratoxin A is the most prevalent and potent of the ochratoxins. Only ochratoxin A, and rarely ochratoxin B, have been found to occur naturally in food and feed. Both OTB and OTC are less toxic and more uncommon than OTA (*107*). OTA's deleterious biochemical effects derive from its structural similarity to the amino acid phenylalanine and thus directly affect the enzymes involved in phenylalanine metabolism (*190*).

OTA Toxicity and Related Health Risks

OTA is a relatively stable molecule that "possesses carcinogenic, nephrotoxic, teratogenic, immunotoxic, and neurotoxic properties" (*70*). After it is ingested, OTA becomes absorbed in the small intestine and becomes tightly bound to molecules in the blood. From there the majority is distributed to the kidneys, with much lower concentrations in the liver, muscle, and fat. Metabolism occurs in bile or the liver. In the former, OTA is hydrolyzed to ochratoxin α

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by intestinal microflora, a metabolite that can be reabsorbed by the intestine. The liver and kidneys are relatively unable to hydrolyze OTA. Instead, in the liver, OTA is oxidized, forming 4-OH-ochratoxin A. OTA is excreted via feces and in urine (*211*). OTA has crossed the placenta in rat and porcine models. It has been found that OTA can transfer to milk in rats, rabbits, and, most notably, humans. The half-life of OTA in blood serum is 510 hours in monkey, 72-120 hours in pigs, and 4.1 hours in chickens. In a single study involving one human the half-life was determined to be 35 days (*211*).

OTA's mode of action has yet to be fully elucidated. OTA inhibits protein synthesis and possibly RNA and DNA synthesis. *In vitro* studies have found OTA to impede enzymes involved in phenylalanine metabolism. This effect can be counteracted by adding phenylalanine or aspartame (*203*). The details surrounding OTA's genotoxicity remains unclear. Further studies are warranted in this area as well as in determining the mechanistic details by which OTA causes nephrotoxicity and carcinogenicity. It is known that embryotoxicity, teratogenicity, and immunotoxicity occur only at doses higher than those that cause nephrotoxicity (*203*).

The no observable effect level (NOEL) of OTA in rats after oral exposure was found to be 21 ng/g (*162*). Short-term toxicity studies have shown lethal doses of OTA to cause hemorrhages, intravascular coagulation, and necrosis of the liver, kidney, and lymphoid organs. Dogs and pigs are more sensitive to OTA than rats and mice (LD_{50}: 0.2, 1, 20-30, and 46-58 mg/kg body weight, respectively) (*211*). The long term effects of OTA exposure include renal tumors and, in some cases, liver cancer (*190*). It is also thought that OTA might lower the immune response in mammals and increase their susceptibility to bacterial infections (*186*).

In humans, OTA has been causally linked to Balkan endemic nephropathy (BEN), a chronic renal condition among those living in the Balkan region (e.g. Bosnia, Croatia, Bulgaria,

Romania, and Serbia) due to the consumption of extremely high levels of OTA in their diet (*126*). Tumors of the urinary tract, renal pelvis, and ureters are highly correlated with BEN (*210*). However, the association between OTA and BEN and between OTA and urinary system tumors remains highly controversial since as of yet no convincing evidence from human epidemiology confirms this association (*203, 210*). More recently, OTA has been linked to human renal disease in Egypt and Tunisia (*119, 202*). In Denmark, OTA has been associated with porcine nephropathy. OTA has been classified as a Group 2B carcinogen by the International Agency for Research on Cancer (IARC), meaning that OTA is possibly carcinogenic to humans (*98*).

Ochratoxigenic Fungi

Ochratoxin A is produced by several *Penicillium* and *Aspergillus* species (Table 1). It should be noted that prior to 1985 ochratoxigenic *Penicillium* were collectively classified as *P. viridicatum* (*33, 69*). Advancements in DNA sequencing, extrolite analysis, and culture media since that time have resulted in the current classification (*72*).

OTA is a secondary metabolite meaning that its production is not essential to survival and thus is produced after the fungus has completed its initial growth phase and has begun the process of sporulation (*35*). OTA has been detected in conidia and at a lesser concentration in the sclerotia, or hardened mass of mycelium, of the fungi (*18*).

The primary OTA-producers on a given commodity largely depends on the climate in which the crop is grown as well as the substrate itself. In cold and temperate climates, such as Europe, Canada, and the United States, *Penicillium* species are the most common OTA producers. *Aspergillus* species may occasionally be isolated from foods in temperate environments but they are much more common in tropical climates (*127*).

Table 1.1. Known ochratoxin-producing fungi.

Genera	Species
Penicillium	*P. nordicum; P. verrucosum*
Aspergillus	
section *Aspergillus*	*A. glaucus*
section *Circumdati*	*A. auricomus, A. cretensis, A. flocculosus, A. melleus[a], A. ochraceus, A. ostianus[a], A. persii[a], A. petrakii[a], A. pseudoelegans, A. roseoglobulosus A. sclerotiorum, A. steynii, A. sulphureus,, A. westerdijkiae, Neopetromyces muricatus[b]*
section *Nigri*	*A. awamori, A. carbonarius, A. lacticoffeatus, A. niger, A. sclerotioniger*
section *Flavi*	*A. albertensis, A. alliaceus, A. lanosus*

[a] Trace producers
[b] Teleomorph
Adapted from: Abarca et al. 1994; Bayman and Baker, 2006; Frisvad et al. 2004; Mateo et al, 2011; Pardo et al, 2006a; Samson et al., 2004; Sánchez-Hervás et al., 2008.

A. ochraceus infects cereals that are grown in tropical and sub-tropical climates, as well as coffee, cocoa, and edible nuts (*150*). *A. carbonarius* is the primary OTA producer in grapes, wine, and vine fruits, however, *A. ochraceus* and *A. niger* also can contribute (*34, 152*). *A. ochraceus* and *A. melleus* were the most common OTA-producing species isolated from Californian figs, tree nuts, and orchards but none of the isolates (N=53) produced greater than the detection limit of 10 ng/g OTA. Six *A. alliaceus* were isolated, all of which produced detectable levels of OTA. Although *A. alliaceus* is rare (found in <0.008% figs and <0.1% pistachios), this organism may be more important role than *A. ochraceus* in terms of OTA contamination in California figs, which is contrary to previous thought (*19*).

Aspergillus section *Circumdati* (e.g. *A. westerdijkiae, A. steynii*) are important OTA producers in Mediterranean crops such as dried fruits, nuts, coffee and cocoa beans (*116*). *Aspergillus* section *Nigri* are routinely associated with OTA production on tropical and sub-tropical foods (e.g. grapes, dried fruits) and are highly resistant to ultraviolet (UV) light and high temperatures, the presence of acid, and low water activity (a_w) (*116*). In a study of Spanish cocoa

beans, the vast majority (83.8%) belonged to *Aspergillus* sections *Flavi* (*A. flavus* and *A. tamarii*) and *Nigri* (*A. niger* and *A. carbonarius*) (*168*).

The primary ochratoxigenic species in stored Argentinian corn (N=50) were *A. flavus*, *A. niger* var. *niger*, *A. niger* var. *awamori*, *A. japonicas* var. *japonicas* (*125*). In Argentinean peanuts *A. carbonarius* was the primary OTA producer (*124*).

P. verrucosum primarily contaminates cereals but has been isolated from cured ham and brined olives on occasion (*46, 91*). *P. verrucosum* is the only OTA producer to date in cereals and cereal products in temperate climates such as in northern Europe and Canada (*A. ochraceus* may be found in temperate climates but rarely) and more recently in countries with warmer climates such as Italy, Spain, France, and Portugal (*117*).

Proteinaceous and high salt (>6% sodium chloride) foods such as fermented or dried meats and fish and salted cheeses are commonly contaminated by *P. nordicum* (*18, 28, 81*). Sonjak et al. (*183*) found ochratoxigenic *P. nordicum* to be a contaminant of sea salt, an ingredient used in the production of cured meats, indicating that perhaps salt may be the means by which this organism contaminates these products. *P. nordicum* is considered a more consistent and productive OTA producer compared to *P. verrucosum* (*173*).

Co-Occurrence of OTA and Other Mycotoxins

Mycotoxigenic fungi tend to be very competitive, dominating mycoflora under optimal environmental conditions, and ochratoxigenic fungi are no exception (*127*). Species belonging to *Aspergillus* section Circumdati are characterized by their ability to produce at least one of the following extrolites: penicillic acids, xanthomegnins, melleins, or ochratoxins (*72*). *A. ochraceus* has the ability to produce OTA and penicillic acid concurrently (*75*). It has also been known to produce asperlactone, isoasperlactone, mullein, and hydroxymellein (*15*).

Most *Penicillium verrucosum* isolates produce the following toxic metabolites: OTA, OTB, citrinin, verrucolones (PC-2, LL-P888γ, verrucosapyrone A and B), and verrucins A, B, C, D, and E (*75*). Of the aforementioned toxins, OTA and citrinin are the most important in terms of human health significance. The structure of citrinin is similar to the dihydroisocoumarin moiety of OTA. However, it does not contain a chloride (*173*). Upon examination of 86 *P. verrucosum* isolates, investigators found that 66% produced ochratoxin A, 87% produced citrinin, 92% produced verrucin and 100% produced verrucolone (*75*). *P. nordicum* isolates (N=20) produced ochratoxin A, verrucolones, anacines, and sclerotigenin (*112*).

Knowing which types of fungi are present on a food commodity is important in terms of the potential interaction and competition between toxigenic and non-toxigenic species (*127*). Many ubiquitous penicillia species (*Penicillium* section Viridicata) are known to produce penicillic acid, which acts synergistically with OTA in terms of nephrotoxicity (*75, 187*). Baydar et al. (*17*) found that 24/25 (96%) of Turkish retail cereal- and pulse-based flours and starches tested contained detectable levels of OTA. All of those samples also were positive for aflatoxin. Conversely, Shotwell et al. (*178*) analyzed 848 wheat samples from the United States for OTA and aflatoxins. While 11 samples contained OTA (Limit of detection [LOD] = 15-30 ng/g), none of the samples had detectable levels of aflatoxin (LOD = 1-3 ng/g).

A study conducted on stored barley samples (N=105) analyzed for aflatoxins and OTA found co-occurrence in 4.8% of the samples (*127*). Co-occurrence of OTA and deoxynivalenol (DON) was found in 41.5% (N=106) of beer samples collected from 25 European countries (*23*). Zinedine et al. (*217*) analyzed corn samples for co-occurrence of OTA, fumonisin B_1, and zearlenone. Eight (40%; N=20) samples had detectable levels of OTA, all of which were also contaminated with fumonisin B_1. All three mycotoxins co-occurred in only one sample.In a study

of 46 breakfast cereals originating from a Spanish market that were analyzed for the presence of aflatoxins, OTA, and zearalenone (ZEA), it was found that both OTA and ZEA were detected in 28% of the samples (*96*).

OTA Biosynthesis

The pathway in which OTA is synthesized is not well known although several possible pathways have been proposed (*87, 93*). Thus far an alkaline serine protease (*asp*PN), nitrate transport protein (*ntra*PN), polyketide synthase (*otapks*PN), non-ribosomal peptide synthetase (*otanps*PN), chloroperoxidase (*otachl*PN) and a transport protein (*otatra*PN) have been identified as involved in OTA biosynthesis (*81*). The OTA polyketide synthase (*otapks*PN) and the non-ribosomal peptide synthetase (*otanps*PN) genes are considered the two primary genes encoding important enzymes in OTA biosynthesis (*3, 28, 87*). Gallo et al. (*77*) found that a second polyketide synthase gene, *aoks1*, is also required for OTA production in *A. westerdijkiae*.

In general, the pentaketide isocoumarin group is believed to be formed from acetate and malonate via a polyketide synthesis pathway and thus requires a polyketide synthase (PKS) enzyme (*132*). In *A. steynii* (formerly identified as *A. ochraceus*) isocoumarin is then carboxylated and then chlorinated by chloroperoxidase or a halogenase enzyme to form ochratoxin α. The phenylalanine moiety is synthesized by the shikimic acid pathway (*140*).

The final step, which is catalyzed by a non-ribosomal peptide synthase (NRPS), involves linking the isocoumarin moiety to the phenylalanine moiety by a carboxyl group. It is thought that OTB may be formed when chlorine is lacking and when OTA becomes dechlorinated (*87, 141*). Recent work by Gallo et al. (*77*) has further elucidated the latter steps of the process. A compilation of the various OTA biosynthesis pathway hypotheses are depicted in Figure 3.

OTA biosynthesis in *P. nordicum* and *P. verrucosum* are very similar, with the exception of the gene encoding polyketide synthase (*81*). The two OTA-producing Penicillia differ in that *P. nordicum* is positive for both *otapks*PN and *otanps*PN genes while *P. verrucosum* is positive for only the *otanps*PN gene (*28*). Expression of *otapks*PN in *P. nordicum* is low when under acidic conditions (pH <5.0) (*79*). Another polyketide synthase in *P. verrucosum* known as *otapks*PV has been reported. It is highly regulated by environmental stimuli (*171*).

O'Callaghan et al (*140*) observed that *otaE* (oxidoreductase) and *otaT* (transporter) were co-expressed with *otapks*PV during OTA production. The aforementioned results gave rise to the identification of a gene cluster encoding an oxidoreductase (OtaE), a polyketide synthase (OtapksPV), and a transporter protein (OtaT) (*140*).

Similar to *Penicillium*, the *otanps*PN gene is also required for OTA synthesis in *Aspergillus*. Therefore this gene has been targeted in multiple studies as a way to quantify OTA-producing fungi regardless of species (*28, 81, 164, 165*).

OTA Production as an Adaptation Strategy

Studies conducted on *Fusarium* concluded that the regulation of genes involved in toxin biosynthesis is closely controlled by growth conditions (*155, 161*). This relationship has also been shown to be true for ochratoxigenic fungi. It has been found that polyketide synthase gene expression of several OTA-producing species is regulated by a variety of extrinsic factors, both under optimal growth conditions and under mild stress conditions such as sub-optimal water activity or in the presence of low preservative concentrations (*171*). Although the specific conditions under which fungi produce OTA are not all known there are several studies that show the ability increases the organism's survival. According to Dowd (*52*) 2,500-25,000 ng/g OTA are toxic to corn earworm and fall army worm larvae, causing weight loss and death.

Figure 1.3. Compilation of the various OTA biosynthetic pathway hypotheses*.
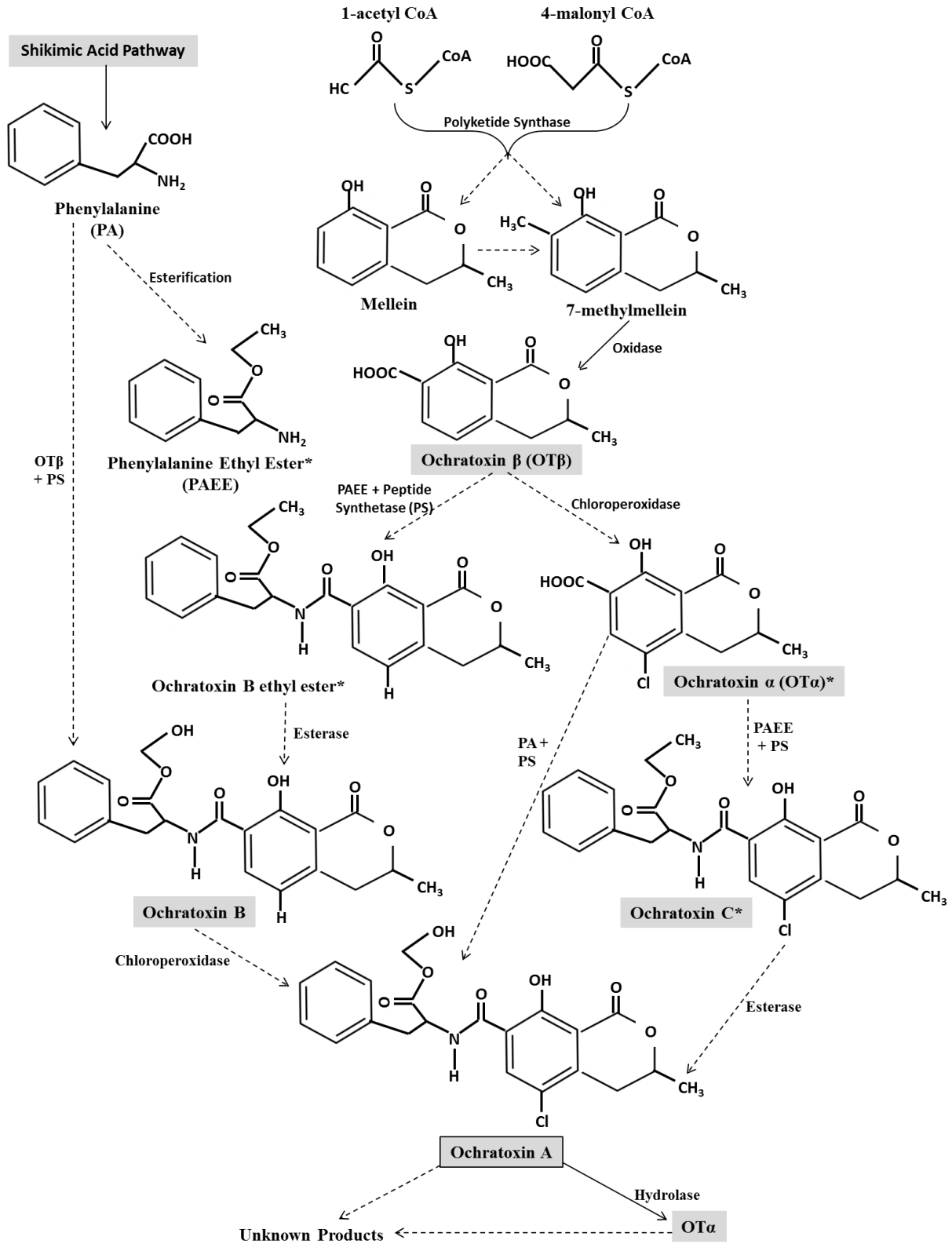
*Asterisks and dotted lines denote hypothetical intermediary compounds and pathways, respectively. Adapted from Gallo et al., 2012.

Recently it has been elucidated that it may impart a means by which ochratoxigenic *Penicillium* species can adapt to environments that are rich in sodium chloride (*173*). In such an environment osmotic stress is detected and osmotic-sensitive signal cascades induce OTA biosynthesis. Halogen ions are incorporated into OTA, a means by which to excrete chlorine out of the cell, thus reducing the inhibitory affect that chloride salts have on fungal growth. This adaptation allows the fungi to remain viable and increase its competitiveness (*173*).

Factors Influencing OTA Production

The level of fungal growth and subsequent OTA contamination that occurs on any given commodity depends on the environmental conditions (e.g. temperature, CO_2, water activity), the OTA-producing strain, the endogenous microflora in the commodity, as well as the commodity itself (*34, 97, 106, 168*). In order for cereal grains to be colonized, spore germination must first take place. Based on observations made of *A. ochraceus* and *P. verrucosum* inoculated onto media, the minimum water activity (a_w) required for spore germination increased with decreasing temperature, the lag time before germination is more sensitive to the water activity level as temperature decreases, and germination rates decrease with decreasing water activity and temperature (*150*).

A. ochraceus can grow at temperatures between 8-37°C, with the optimal temperature between 24-31°C whereas *P. verrucosum* is characterized by growth at 0-31°C, with an optimum at 20°C (*99*). Both organisms require a narrower a_w range for mycelial growth, as compared to spore germination. Optimal conditions for mycelial growth of *P. verrucosum* are 20°C and 0.95-0.99 a_w (*150*). *P. verrucosum* can grow down to 0.80 a_w (*53*). It should be noted that temperature and water availability necessary for fungal growth does not always directly correspond to the

conditions required for OTA production (*150*). Similarly, the presence of ochratoxigenic species alone is not an indicator that OTA is also present.

Environmental Conditions

OTA production is strongly dependent on environmental conditions (*127*). The most important factor is water availability, which directly relates to moisture content (MC) (*106*). The minimum a_w of a system required for OTA production is higher, and narrower in range, than that required for growth of the organism. Temperature also influences fungal growth and OTA production (*149*).

Cairns-Fuller et al. (*34*) completed a study examining growth of *P. verrucosum* and *A. ochraceus* and OTA production in wheat stored for 56 days under several moisture and temperature combinations. At 15°C and 20°C OTA was not produced by either of the organisms at 0.80 a_w. Increasing a_w to 0.85 resulted in some OTA production. The most OTA was produced when wheat was stored at 10-25°C and 0.93-0.98 a_w (23.5-27.4% MC). Results showed that 25°C and 0.95 a_w were the optimal conditions for OTA production by *P. verrucosum* in their study. Both studies showed that wheat with a water activity below 0.80-0.83 (17-18% MC) prevented growth of and OTA production by *P. verrucosum* and *A. ochraceus* (*34*). Lindblad et al. (*114*) had slightly different results, finding that under some conditions *P. verrucosum* can grow at 0.80 a_w. Frisvad and Samson (*71*) stated that growth of *P. verrucosum* can occur between 0.81-0.83 a_w and OTA production at 0.83-0.90 a_w. Based on the aforementioned findings, the threat of OTA contamination remains uncertain in grain that is stored between 0.81-0.83 a_w.

A study by Palacíos-Cabrera et al. (*148*) observed that a greater amount of OTA was produced by *A. ochraceus* in raw coffee when exposed to alternating temperatures during storage, as opposed to constant temperature. It was suggested that the result was due to moisture

gradients that formed in the coffee, creating a more favorable environment for OTA production.

Table 2 summarizes key environmental parameters conducive to OTA production by select

ochratoxigenic species.

Table 1.2. Environmental conditions for OTA production by important ochratoxigenic species.

Parameter		Ochratoxigenic Fungi			
		A. carbonarius	*A. niger*	*A. ochraceus*	*P. verrucosum*
Temperature (°C)	Min.	5-15	6-15	5-10	4-10
	Max.	30-45	35-47	30-40	21-31
	Optimal	15-30	15-35	20-35	24-25
Water activity (a_w)	Min.	0.85-0.94	0.90-0.95	0.85-0.90	0.80-0.83
	Optimal	0.95-0.99	0.95-0.99	0.95-0.99	0.95-0.99
Time (days)	Min.	2-5	3-7	3	7
	Optimal	10-15	5-30	9-21	>14

Adapted from Amézqueta et al., 2012; Esteban et al., 2004; Kozakiewicz and Smith, 1994; Magnoli et al., 2007a; Pardo et al. 2004.

Gas composition is another factor. As ochratoxigenic fungi require air to function,

reducing oxygen content can slow or prevent growth. Cairns-Fuller et al. (*34*) reported that for

safe storage of moist cereals >50% CO_2 concentration needs to be reached to prevent OTA

contamination. In another study *P. verrucosum* growth and OTA production was highest in air,

followed by 25% and 50% CO_2 (*34*).

Time is required in most cases in order to achieve a significantly high level of OTA.

Cairns-Fuller et al. (*34*) reported that it would take a minimum of 14 days for irradiated wheat

grain stored between 15-25°C for OTA produced by *P. verrucosum* to begin accumulating. The

same study demonstrated that the CO_2 level and a_w of the substrate together had an "enhanced

inhibitory effect" on growth and OTA production by *P. verrucosum* on irradiated wheat. Under

optimal a_w levels, an increase in CO_2 levels can inhibit growth. When a_w is reduced to less

optimal levels and CO_2 is increased, an even greater reduction in fungal growth and OTA

production is achieved. This interaction was not synergistic, however (*34*).

OTA production is also strongly influenced by pH. Levels >7.0 inhibit OTA production whereas pH < 7.0 are conducive for OTA production (*141*). Schmidt-Heydt et al. (*171*) observed that mild stress conditions activated OTA biosynthesis. When *P. verrucosum* was grown in a medium (a_w = 0.98 or 0.95) containing the preservatives calcium propionate or potassium sorbate, colony growth decreased as preservative concentration increased. At the same time, OTA production was stimulated at sub-optimal a_w (0.95 and 0.93) (*171*).

Another environmental condition that affects OTA production includes light exposure. Schmidt-Heydt et al. (*172*) reported that *P. nordicum* shifts from OTA production to OTB when the organism is exposed to bright light. In another study the effect of photoperiod on OTA production by *A. carbonarius* was investigated by Belli et al. (*21*). No significant effect on OTA production was observed, although the light/dark cycles did enhance fungal growth.

Knowing the optimal and minimal requirements for each abiotic factor will aid in optimizing post-harvest storage conditions of commodities (*150*). However, outside the laboratory the actual climactic conditions in an organism's microenvironment, by way of rainfall, temperature, and other factors, is dynamic and constantly under flux. These changes affect OTA production; however, predicting the amount of OTA produced on a given commodity as a result of certain climatic parameters remains elusive. General trends have provided some level of prediction. Regions that are generally wet as opposed to dry regions have higher incidences of OTA contamination. Also, depending on the ochratoxigenic species, a warmer or cooler climate might be more conducive for OTA production (*106*). The exact temperature and a_w optimum for OTA production varies depending on the specific ochratoxigenic isolate (*149, 150*).

Ochratoxigenic Isolates

Studies have demonstrated that the ability to produce OTA can be highly variable among isolates of the same species (*151*). This was supported in a study by Frisvad et al. (*75*) when only 16% of amplified fragment length polymorphism (AFLP) haplotypes of *P. verrucosum* isolates tested included >1 isolate. It is believed that the genes required for producing OTA are present in all *P. verrucosum*, but that in some cases it is silent or that it requires specific environmental conditions to be expressed (*18, 75*). The *A. ochraceus* group has been divided into two clades based on mitochondrial DNA restriction profiles. The first clade is made up of OTA non-producers. The second clade contains both OTA producing and non-producing members (*198*).

According to Amézqueta et al. (*11*) *P. verrucosum, A. ochraceus, A. niger,* and *A. carbonarius* are the most significant OTA-producing species due to their high prevalence in the commodities they affect and the frequency of OTA-producing strains. A study by Frisvad et al. (*74*) reported that 74% (N=321) of *P. verrucosum* barley, wheat, and oats isolates were OTA producers. Larsen et al. (*112*) found that 79% of *P. verrucosum* isolates tested (N=48) produced OTA. Seventeen *P. verrucosum* isolates were obtained from Spanish white wheat flour samples. OTA production was confirmed in 64.7% of the isolates (*32*).

A review by Amézqueta et al. (*11*) compiled results of 4 studies that reported prevalence of OTA producers isolated from cereal. Overall, 15-100% of *A. ochraceus* isolates tested were confirmed OTA producers. In stored corn (N=50), 1 (25%) of 4 *A. ochraceus* isolates produced OTA. Among the other *Aspergillus* isolates 25% (n=30) were OTA producers (*125*). Collectively, several studies on green coffee have reported 75-90% of the *A. ochraceus* isolates were capable of detectable OTA production (*150*).

A. carbonarius (section *Nigri*) is considered to be the most ochratoxigenic species in Argentinian dried vine fruits given that 96% of the isolates tested were OTA producers whereas only 1% and 0% of the isolated *A. niger* and *A. japonicus* strains, respectively, produced OTA. Even though *A. carbonarius* were frequently isolated, the OTA concentration on the fruit was much less than what the isolates produced in pure culture. These differences are likely attributable to environmental conditions during the growth of the fruit, harvest conditions and practices, as well as storage practices that were not conducive for OTA production by this species (*166*).

A total of 420 fungal strains were isolated from Spanish cocoa beans. The vast majority (83.8%) belonged to *Aspergillus* sections *Flavi* (*A. flavus* and *A. tamarii*) and *Nigri* (*A. niger* and *A. carbonarius*). Of the 138 strains from *Aspergillus* section *Nigri,* 47.1% (n=65) were confirmed OTA producers. All of the *A. carbonarius* strains (n=6) and 44.7% (n=132) of the *A. niger* strains produced OTA (*168*).

Abarca et al. (*1*) found that the number of suspect OTA-producing *Aspergillus* section *Nigri* isolates produced OTA *in vitro* between <2% to >90% of the time, depending on the species and conditions used. Other reports have found almost 100% of *A. carbonarius* and *A. steynii* isolates to be confirmed OTA producers (*83, 130*). In a compilation of 3 studies, 6-75% of *Aspergillus* section *Nigri* isolates produced OTA. Similar to the aforementioned studies, the authors suggested that differences in culture conditions (i.e. time, temperature, isolation medium) used most likely had an effect on the results and would account for some of the difference observed between studies (*11*).

A few studies have suggested that the level of *P. verrucosum* contamination in a sample can be used as an indicator of OTA contamination. In irradiated barley samples a decrease in the

number of *P. verrucosum* due to competition with other fungi led to a decrease in OTA levels. This correlation was especially strong when *P. verrucosum* was growing in pure culture or in competition with only one other fungus. Overall, the correlation between seed infection rates and OTA production was slightly higher than that of colonization and OTA production. The authors point out that aforementioned correlations were not consistent when *P. verrucosum* was in a mixed culture and thus the reported observations have limited applicability in complex ecosystems, such as stored grain. Seed infection rates and colonization rates were significantly correlated in the majority of the treatments at 20°C or 30°C and at 0.90 or 0.95 a_w. The authors conclude that although predicting the exact OTA concentration in a sample is dependent on numerous factors, *P. verrucosum* colonization may be a good indicator of OTA contamination (*163*).

Lund and Frisvad (*117*) reported that samples with >7% *P. verrucosum* kernel infestation indicated OTA contamination. However, no direct correlation between these two factors was established. Lindblad et al. (*114*) established a logistical model using inoculated and stored winter wheat that can be used to predict OTA contamination given a known level of *P. verrucosum* in a sample as well as environmental factors such as temperature (10-25°C) and a_w (0.80-0.95). The general trend was that the probability of reaching noncompliant OTA levels increased with increasing a_w and *P. verrucosum* concentration. Furthermore, the rate of probability increases as a_w increases. There was no significant effect of storage temperature on the risk of OTA production.

In naturally contaminated wheat, rye, oats, and barley samples (N=220) *P. verrucosum* was detected in 26% of the samples and 4% exceeded 5 ng/g. Overall, a level of 10^3 colony forming units (CFU)/g of *P. verrucosum* was suggested to be the limit at which there was a

probable risk that the sample contained ≥5 ng/g OTA regardless of the a_w. Samples with low levels of *P. verrucosum* that exceeded 5 ng/g were found among both artificially and naturally contaminated samples, which affected the accuracy of the model's predictions. The authors suggest that lowering the threshold to 10^2 CFU/g would compensate for this observation by decreasing the risk of overlooking positive samples. The downside of the approach would be the increase in the number of samples that would require analysis for OTA (*114*).

Substrate

OTA production is also influenced by differences in the nutrient profile and physical structure of cereal grain as well as by competition with other microorganisms (*40, 54*). Glutamic acid and proline have been found to induce OTA production by *A. ochraceus* in culture. Thus, a high content of these amino acids could be a cofactor in OTA production (*67*).

It has been suggested that the level of OTA production depends on the carbon source, with sucrose providing the highest levels. OTA is produced at much lower levels if the main carbon source is glucose and at even lower levels in the presence of fructose or lactose (*68, 133, 152*).

Recent studies using *P. verrucosum* grown on agar medium showed that particular carbon sources can act to increase or decrease OTA production. The following carbon sources, all of which caused an increase in OTA production compared to OTA production on the medium alone, are listed in order from having the greatest to the least effect: galactose (32-fold increase), glycerol (19-fold increase), succinate, lactose, and maltose. Glucose had an insignificant effect on promoting OTA production when added alone. In the presence of galactose the addition of glucose resulted in a 10-fold repression of OTA production (*140*). Another study reported that

decreasing the amounts of glucose in a substrate effectively increases OTA production by *A. ochraceus* (*133*).

Abbas et al. (*3*) observed a complicated mix of results when using OTA-restrictive and OTA-permissive media. OTA production by *A. ochraceus* has been found to be differentially regulated by a number of culture conditions including pH and the nutritional profile of the growth medium (*3, 141*). Different carbon and nitrogen sources have different effects on OTA production *in vitro*. In an OTA-permissive medium, glucose, sucrose, maltose, galactose, xylose, and glycerol all repressed OTA production. However, in an OTA-restrictive medium, glucose, galactose, sucrose, glycerol and lactose relieve the repressive effect. On both OTA-restrictive and OTA-permissive media, the addition of lactose or galactose increased OTA production, although lactose had a greater effect than galactose. These results indicate that the abiotic factors that affect OTA synthesis are complex. The group also found that organic nitrogen sources (e.g. urea, amino acids [glutamine, phenylalanine, lysine, proline]) induce OTA production. Ammonium chloride was found to markedly reduce OTA production (*3*).

It has been found that different varieties of wheat, barley, and rye demonstrate different levels of resistance to OTA production by *A. ochraceus* (*40*). In comparing cereal species, overall, rye kernels had the highest levels of OTA, followed by wheat and then barley. Similarily, data reported in 2002 by the European Commission as a result of a survey of cereal grains found rye (N=444) to contain the highest average (0.60 ng/g) and maximum level (33 ng/g) of OTA when compared to wheat (N=979), barley (N=142), maize (N=267), and oats (N=164) (*102*). OTA was found in higher concentrations on the external surface of rye kernels as compared to barley and wheat. In the latter OTA was more abundant inside of the kernel (66-

>90%) than on the outside of the kernel. This difference has been attributed to seed coat thickness (*40*).

Subsequent studies have supported the finding that rye is the most sensitive of the small grain cereals in regards to *P. verrucosum* and OTA formation (*58, 103, 104*). A study by Elmholt and Rasmussen (*61*) found that spelt samples had higher percentages of *P. verrucosum* contamination as compared to rye although the differences were not significant. Spelt was significantly more contaminated with *P. verrucosum* than wheat, barley, and oats prior to drying.

Chelkowski and Cierniewska (*39*) note marked differences between fungal invasion that took place on non-viable (i.e. autoclaved) kernels versus viable kernels. In the former fungal mycelium overtook the kernel in only a few days, covering all surfaces, and subsequently producing spores. On the other hand, it took a few weeks for viable kernels to be infected even when subjected to high inoculum concentrations (*39*). The same study confirms findings from two previous studies that OTA was typically found in highest concentrations in the nutrient-rich aleurone layer of the invaded kernel (50-95%) and to a lesser extent on the surface and in the innermost layers (*39, 40, 42*). Ibáñez-Vea et al. (*96*) found that OTA, as well as other mycotoxins, generally accumulated on or just beneath the epidermis of the grain. In their study the group recovered 50% of OTA from the outside of the barley grain. Osborne et al. (*146*) reported that a greater proportion of OTA was found in the bran and offal fractions of hard wheat in comparison to soft wheat.

An additional finding was that OTA concentration was 2-3X higher on autoclaved kernels versus viable kernels incubated for the same length of time (*40*). It is thought that the autoclaving process disrupts the zinc-phytic acid complex, thus freeing zinc for utilization by fungi. The effect of zinc was supported under controlled conditions in which zinc was added at

1000 ng/g to viable barley kernels. OTA production increased as compared to untreated kernels of the same barley variety (*40*).

Not only are there differences in susceptibility to OTA between cereal grain types, but cultivar differences have also been reported. Elmholt and Rasmussen (*61*) found out of 4 spelt cultivars, all of which had been grown in the same field and had similar MC, 2 contained 18 and 92 ng/g OTA, whereas the other 2 were contaminated at 0.1 and 0.2 ng/g OTA.

Valero et al. (*192*) demonstrated the effects of endogenous microflora on OTA production. They found that in the presence of microbial competition, such as non-toxigenic *A. niger*, OTA production by *A. carbonarius* on grapes was inhibited at 30°C but not at 20°C, the organism's optimal temperature for OTA production. This result observed at 30°C was linked to multiple factors including: growth restriction, consumption of specific nutrients required for OTA synthesis, other fungi degrading OTA that is produced, and excretion of compounds that block OTA synthesis by endogenous fungi (*192*).

Overall, the environment and substrate dictates the OTA producing species and the amount of OTA production (*106*). More research is needed to understand the effects of each of these variables on OTA production in the field as the majority of the work conducted to date has been in the laboratory setting. Khalesi and Khatib (*106*) have stated that future models of ochratoxigenic fungi growth and OTA production should be based on data that reflects natural variability observed in real life.

OTA in Commodities and Food

Ochratoxin A is ubiquitous and consumed at low levels on a daily basis by a majority of the human population. In a study by Health Canada it was found that 100% of human sera tested had detectable levels of OTA (*89*). In 2001, the Joint FAO/WHO Expert Committee on Food

Additives (JECFA) estimated the mean total OTA intake for a European weighing 60 kg to be 45

ng/kg body weight per week. Based on this estimation, a consumer who falls in the 95th

percentile for cereal consumption alone would approach the provisional tolerable weekly intake

of 112 ng/kg body weight per week (*210*). OTA has been found worldwide in animal feed and a

variety of commodities and foods (Table 3). Humans can ingest OTA either directly from foods

tainted with OTA or by consuming meat or milk from animals fed with OTA-contaminated feed

(*56*).

Table 1.3. Commodities and foods naturally contaminated with ochratoxin A by source.

Category	Affected Commodities/Foods
Plant	Grapes, dried vine fruits (raisins, figs), tree nuts (pistachios, almonds, walnuts), coffee (green and roasted), cocoa beans (chocolate), spices, oil seed, rice, legumes (peanuts, soy beans, garbanzo beans, dried peas), olives
Cereals & Cereal Products	Wheat, rye, barley, maize, oats, infant cereal, flour, dry pasta
Animal	Pork and poultry (blood/meat)
Beverage	Wine, beer, grape juice, coffee, cocoa, cow milk, infant formula

Adapted from: Abarca et al., 2001; Aziz et al., 1998; Cabañes et al., 2010; Geisen et al., 2006; Ibáñez-Vea et al., 2011; Imperato et al., 2011; Jørgensen, 1998; Kuiper-Goodman and Scott, 1989; Magnoli et al., 2007b; Murphy et al., 2006; Ng et al., 2009; Pardo et al, 2006a; Pitt, 2000; Sánchez-Hervás et al., 2008; Shotwell et al., 1969; Solfrizzo et al., 1998; Zimmerli and Dick, 1996.

Cereals and cereal-based products are the most significant daily source of OTA in the

human diet (50-80%). Wine is another substantial source (~15%) followed by coffee (~12%)

(*48*). A study published by the European Commission that examined OTA levels in various

ingredients and food products at retail reported that spices (N=361) contained an average of 1.2

ng/g OTA, beer (N=496) averaged 0.03 ng/g, cocoa (N=547) averaged 0.24 ng/g, roasted

(N=788) and instant (N=226) coffee averaged 0.62 ng/g and 1.3 ng/g, respectively, wine

(N=1470) samples contained 0.36 ng/g on average, dried vine fruits (N=593) had an average of

3.1 ng/g OTA, grape juice (N=146) averaged 0.56 ng/g OTA (*102*). Another survey of beer

(N=106) from 25 European countries found 67.9% were positive for OTA. The median level was 0.009 ng/g (*23*).

Zinedine et al. (*217*) tested 20 samples each of corn, barley, and wheat that were obtained from Moroccan markets. A total of 8 (40%) corn, 8 (40%) wheat, and 11 (55%) barley had detectable levels of OTA. The LOD was 0.01 ng/g.

A small sampling (N=25) of twelve Turkish seed-, pulse-, and cereal-flours and starches were purchased at the retail level and analyzed for OTA (LOD=0.025 ng/g). OTA was detected in 24 (96%) of the samples at levels ranging from 0.31-4.07 ng/g. All of the wheat-based flours (n=12) contained detectable levels of OTA (0.38-2.23 ng/g), the single sample of barley-based flour had 4.07 ng/g OTA, the highest level detected in the sample set. The wheat starch sample had 0.31 ng/g OTA (*17*).

OTA has been detected in breakfast cereals at the retail level in Spain, France, Greece, Turkey, and Canada. In Spain OTA was found in 39% of the samples (n=46). The mean concentration was reported to be 0.37 ng/g (*96*).

Ng et al. (*137*) conducted a survey of OTA in dry pasta (regular, whole wheat, and couscous; N=274) purchased in stores over a period of three years. OTA was detected in 21% of the samples in 2004 (mean = 0.30 ng/g; max=1.9 ng/g), 18% in 2005 (mean = 0.28 ng/g; max = 1.4 ng/g) and 66% in 2006 (mean = 0.76 ng/g; max = 3.3 ng/g). The limit of quantification (LOQ) of the method was 0.5 ng/g (*137*).

A study conducted by Imperato et al. (*97*) found OTA to be the most prevalent (17.6%) mycotoxin in several food products (n=345) imported to Italy. In the same study the highest concentration of OTA detected (23.7 ng/g) was in a green coffee sample imported from Costa Rica (*97*). Vecchio et al. (*198*) report 96% (N=48) of instant coffee samples obtained from

Italian retail stores had detectable levels of OTA (LOD = 0.05 ng/g; LOQ = 0.2 ng/g). The mean and maximum OTA level was 1.27 ng/g and 6.40 ng/g, respectively.

All animals fed grain contaminated with OTA have the potential to contribute to human exposure. OTA exposure causes a decrease in productivity and an increase in mortality in livestock (*54*). Monogastric animals such as pork and poultry are highly sensitive to OTA. As much as 50-60% (dry matter) of their feed consists of cereals and by-products of cereals (*170*). The meat, especially any products containing the blood, liver, and/or kidneys in particular, from animals fed OTA contaminated feed serves as a significant source of human OTA exposure in some populations (*102, 147, 203*). Ruminants are less susceptible to the effects of OTA since bacterial enzymes and protozoan in the ruminal fluid are able to degrade the toxin into ochratoxin α, a less toxic metabolite (*128, 190, 210*). Thus, the by-products of ruminants are not a significant source of OTA.

OTA in Cereal Grains

OTA contamination can occur during multiple stages of the grain supply chain: prior to harvest, during the harvesting process, drying and storage, and during some types of processing (*23, 127*). Although little is known about the life cycle of ochratoxigenic fungi, *P. verrucosum* has been isolated from 16% (N=68) of Danish soil samples and field experiments have shown that *P. verrucosum* conidia are able to survive and grow in field soil for >18 months. This demonstrates the ability of *P. verrucosum* to become a part of the soil ecosystem in some environments, although further studies are warranted (*58, 59*). It has been stated that temperature and water availability are key factors contributing to fungal colonization of commodities (*208*). In addition, plant moisture, drought stress, and insect damage all contribute to increased risk of fungal invasion and the presence of OTA (*10*). Other factors include: the weather before and at

harvest, time before drying, drying efficiency of machinery, physical state of grain, temperature at harvest, fungal competition, and cleanliness of harvesting equipment and transportation vehicles (*176*).

Pre- Harvest

Elmholt (*58*) analyzed combined winter wheat, spring wheat, barley, oats, and rye prior to drying. *P. verrucosum* was isolated in 60% of the rye samples and 53% of the wheat suggesting that the isolates could have originated from the soil during the harvest process or from farming equipment contaminated with conidia. In 1998, 51% (N=35) of combined samples were contaminated with *P. verrucosum* ranging from 0.6-5.8% infestation per sample (*61*). Another study found 82% (N=78) of combined grain samples prior to drying were contaminated with *P. verrucosum*, each at ≤58.7% infestation per sample. Three (3.8%) of the non-dried field samples had detectable OTA levels. The detection of *P. verrucosum* indicates the risk of OTA contamination but no linear correlation between rate of infestation and OTA was established (*62*). Lund and Frisvad (*117*) point out that such a correlation may be hindered by the complexity of the grain microbiome and the microbial interactions that take place on that level. A study conducted by Lund and Frisvad (*117*) suggested that an infestation rate of ≥7% in wheat or barley was indicative of exceeding 5 ng/g OTA in the sample. The aforementioned study was based on a very limited number of samples and thus further investigation is warranted. In a subsequent study, Elmholt and Rasmussen (*61*) repeated Lund and Frisvad's methodology and found that 52% of their OTA-negative samples exceeded 7% infestation. Infestation rate may not be a reliable predictor of OTA level in a sample but the fact remains that early contamination of grain with ochratoxigenic fungi implies a greater risk of OTA production if the grain is not dried and stored properly (*61*).

Based on a limited number of studies, whether a cereal was grown using organic or conventional practices might impact the presence of ochratoxigenic fungi and OTA level. In a soil survey consisting of samples taken from 65 different farms *P. verrucosum* was detected in 35% of soils from organic soil and 7% of conventionally cultivated soils (*75*). Other studies found that the farm, whether conventional or organic, from which a grain sample was taken was a more significant factor contributing to OTA level than the farming type (*50, 103, 104*). This may in part be due to the fact that *P. verrucosum* has been found to become well-established on some farms but not others (*75*).

Preventative measures at the pre-harvest level include Good Agricultural Practices (GAP) such as: reducing the source of fungal inoculum by removing or destroying old plant material in the field, minimizing plant stress by supplying proper nutrition and soil conditions, planning planting so that seeds are not subjected to high temperatures and drought stress during early growth stages, employing a pest management program to minimize fungal infection and insect damage, rotation of crops, avoiding overcrowding plants, and minimizing mechanical damage to crops during cultivation and harvest (*69*).

Harvest

On the farm, primary inoculum sources of ochratoxigenic fungal contamination include combines, dryers, and silos (*131*). The production and occurrence of OTA in grains during storage depends greatly on the condition of the grain at harvest. Harvest practices that can aid in minimizing OTA contamination include harvesting grain at full maturity or at a time that will minimize plant stress, avoid contact with soil during harvesting, and properly clean all harvesting equipment prior to harvest (*69*).

In the past, 3 publications have utilized surveys to examine the occurrence of OTA in barley and/or wheat produced in the United States. The first was published by Shotwell et al. (*180*) in which 848 graded samples of hard red winter (HRW), hard red spring (HRS), and soft red winter (SRW) wheat were analyzed between 1970 and 1973. OTA was detected in a total of 11 samples (1.3%). A total of 3 (1.0%) HRW wheat samples were positive ranging from <15-35 ng/g whereas the 8 (2.8%) positive samples of HRS wheat were higher at 15-115 ng/g. It should be noted that the detection limit of the method employed was >15 ng/g, a relatively low level of sensitivity compared to current methods, and recovery level of 40-60%. It was also concluded that the grade of the grain cannot be used to predict OTA contamination as each grade level had positive samples (*180*).

A more recent paper surveyed 351 samples of dried peas/beans, barley (whole/cereal, malt), green coffee beans, corn (cereals, meal), oats (meal, crackers), rice (whole, cereals), rye flour, wheat flour, and soy-based baby food products. None of the samples had detectable levels of OTA (LOD = 10 ng/g) (*207*). Pohland et al. (*160*) reported in another U.S. survey that 18 of 127 (14.2%) samples of barley were contaminated at levels of 10-40 ng/g and 11 of 848 (1.3%) samples of wheat were positive at levels of 15-115 ng/g. In this survey, barley had the greatest incidence of OTA but the highest concentrations were found in corn.

In Canada, wheat and flours intended for bread (N=59), corn (N=36), and rice (N=17) were monitored for OTA between 1991 and 1995. OTA ranged from <0.5-6.6 ng/g, <0.5 ng/g, and <0.5-3.96 ng/g, respectively. Four (6.8%) of the wheat/flour samples had >5.0 ng/g OTA (*70*). A 9-year Norwegian OTA survey of 547 domestic wheat and oats and imported wheat and rye samples found 0.9% (n=5) exceeded 5 ng/g and 2.2% (n=12) exceeded 3 ng/g OTA (*70*).

In other countries, the incidence of OTA in barley and wheat has been documented to be generally higher. In a Polish study, researchers analyzed 39 samples of wheat and 40 samples of barley. A total of 7.7% of the wheat samples were contaminated at 0.48-1.20 ng/g (mean = 0.83 ng/g). Similarly, 7.9% of the barley samples were contaminated, but at slightly higher levels than the wheat (6.7-57.0 ng/g, mean = 25.73 ng/g) (*50*). Birzele et al. (*26*) collected German winter wheat samples in 1997 (n=14) and 1998 (n=29) and tested them for OTA directly after harvest (LOD = 0.4 ng/g). Two samples (14.3%) were positive in the first year and 7 (24.1%) in the second year. OTA concentrations ranged from 0.6-0.8 ng/g.

Imperato et al. (*97*) surveyed durum wheat being imported into Italy for OTA in 2008 and 2009. Results showed that none of the samples were positive for OTA (LOD = 0.65 ng/g). Another study examined both barley (n=20) and wheat (n=20) purchased from markets in Morocco. Forty percent of the wheat and 55% of the barley samples had detectable levels of OTA (LOD = 0.02 ng/g). The average contamination level in wheat and barley was 0.42 ng/g and 0.17 ng/g, respectively. The maximum levels reported were 1.73 ng/g in wheat and 0.80 ng/g in barley (*217*). In 1998, corn (n=9) and barley (n=14) samples destined for use as animal feed were collected from two northern Iranian provinces and analyzed for OTA and aflatoxins. None of the barley samples had detectable levels (>0.24 ng/g) of OTA. One corn sample was positive at a level of 0.35 ng/g OTA. The same sample also was positive for aflatoxin (85.30 ng/g) (*212*).

The Danish food-monitoring system has screened for OTA in Danish wheat and rye grain and flour over a period of 14 consecutive years (*103, 104*). A total of 475 wheat samples were tested between 1986 and 1992 of which 135 (28.4%) had detectable levels (>0.05 ng/g) of OTA. Nine samples (1.9%) had ≥5.0 ng/g OTA (*104*). Between 1992 and 1999, 419 wheat samples were tested and 191 (45.6%) were detected at >0.01-0.08 ng/g. Four samples (0.95%) had ≥5.0

ng/g OTA (*103*). Overall, 326/894 (36.5%) had detectable levels of OTA and 13 (1.4%) exceeded 5 ng/g OTA.

The authors state that in 1986-1992 there was a clear relationship between OTA concentrations and weather conditions in that average to wet years resulted in higher OTA concentrations than in dry and very dry years (*104*). Between 1992-1999 the correlation was less clear as the harvest years between 1992 and 1997 were characterized as average to very dry. Only 1998 was considered to be a wet harvest year. When comparing the OTA levels in 1998 with the wet years between 1986 and 1992 it was found that OTA levels were lower in 1998 than in previous wet years. This difference was attributed to improved grain-drying practices that had been implemented after issues with wet grain and the occurrence of OTA became evident in the mid-1980s (*103*). Given that OTA production in grain is a dynamic system, studies with the intent to elucidate the exact nature of the relationship between weather parameters during the planting, growing, and harvest seasons and OTA incidence over time are needed.

Post-Harvest

In the case of cereals, the critical points after harvest are drying, storage, and processing (*10*). Several factors affect OTA production in stored grain. These include: water activity, grain temperature and aeration, the initial concentration of OTA producing fungi, microbial interactions, mechanical damage, and insect infestation (*143*). Cleanliness of storage containers, absence of structural leaks, condensation, temperature and time are all factors that may influence the presence and level of OTA contamination (*176*). Drying and storage facilities and materials contained within should be inspected for locations in which ochratoxigenic fungi survival or even growth may be supported. Fungal conidia and the risk of future contamination can be greatly reduced by implementing through cleaning measures such as vacuum cleaning (*75*).

Drying. If the moisture content can be controlled and maintained at the proper levels after harvest then fungal growth and OTA production can be prevented (*150*). Cereal grain is often harvested at 16-20% MC (*121*). Ideally, small grains should be dried to ≤14-14.5% MC (≤0.70-0.75 a_w) immediately after harvest and provided sufficient air circulation and temperature and moisture control during storage in order to negate fungal growth and mycotoxin production (*120, 176*). Even though it has been shown that ochratoxigenic fungi cannot grow in <17% MC wheat, and that there is little risk of OTA production, if the wheat is not sufficiently dried to <14.5% MC xerophilic molds can grow (*114, 122*). Fungal growth can increase the MC of the grain, making it a more hospitable environment for ochratoxigenic fungi (*122*). Moisture contents at >17% significantly increases the risk of OTA production (*61*). Barley at a_w <0.80 (~14% MC) and at temperature ≤10°C does not support fungal growth (*150*). Malting barley should be dried to 13% MC in order to ensure seed viability (*156*).

Grain is dried using either heated or ambient air. The former is preferred as ambient drying can result in slow drying, with a moisture front moving up through the grain bed. This situation can cause layers of grain to become moldy, thus increasing the chance of OTA contamination (*121*).

In Sweden, a survey found that common issues during drying that lead to an increase in mold contamination included "low fan capacity in near ambient dryers, low drying capacity in heated air dryers, lack of moisture meters, perforations in sealed silos, and defective alarm units on applicators for propionic acid". In order to ensure an accurate MC reading, all moisture reading equipment should be calibrated and a representative number of samples from several points of each load should be tested (*69*).

Storage. Grains are often stored for a length of time prior to being sold and processed. Grain that has been stored without sufficient drying, has been stored too long or stored without proper air circulation has increased likelihood of OTA contamination (*146*). Grain storage facilities should be properly maintained and subject to a sanitation program. If previously stored crops were contaminated with mold, the facility should be chemically treated. The storage structure should be dry, well-ventilated, and free of pests (i.e. rodents, birds, and insects). Low storage temperatures are preferred and temperature should be routinely monitored throughout storage as an increase in temperature can promote mold growth (*69*).

Previous studies have examined the effect of storage on OTA production in grain both by acquiring grain directly from storage facilities as well as by artificially imparting pre-determined environmental conditions on irradiated or non-irradiated grain samples in a controlled laboratory setting. In a Spanish study 105 samples of stored barley were collected over a 2-year period from 21 different elevators. Results showed that 20% of the samples were positive containing 0.05-1.6 ng/g (mean = 0.47 ng/g). The maximum level detected was 2.0 ng/g. In this survey all of the positive samples had been harvested in the spring and then subsequently stored during the summer. The LOD and LOQ were 0.05 and 0.17 ng/g, respectively (*127*).

In storage studies, Abramson et al. (*7*) found that wheat and barley stored for 16 weeks at 15% initial moisture content (IMC) did not result in detectable levels of OTA. At 19% IMC OTA was detected in barley at 20 weeks (70 ng/g) but was not found in wheat. In a separate study durum wheat was stored at an IMC of 16% or 20% at 22°C in a granary for 20 weeks. OTA was detected at 4 weeks with maximum concentration of 6,500 ng/g being produced at 20 weeks. OTA was not detected in the 16% IMC samples (*6*). Birzele et al. (*26*) observed similar results after storing German organic winter wheat at suboptimal temperature (20°C) and different

initial moisture contents (17% or 20%). The samples that had been stored at 17% IMC resulted in no detectable OTA after 6 weeks. In 1997 the OTA level in the 20% IMC samples was 1.2 ng/g after 2 weeks and 1.4 ng/g after 4 weeks of storage. However, in the following year, OTA was not detected in the 20% IMC samples after 6 weeks of storage. The limit of detection was 0.4 ng/g OTA. In 1997 and 1998 the samples that had been stored at 17% IMC had no detectable level of OTA after 6 weeks (*26*).

In another study, Canadian barley, western red spring wheat, and western oats were stored at 21% IMC. By 4 weeks both the barley and wheat had detectable levels of OTA. After 20 weeks of storage OTA was still undetectable in oats. Final OTA concentrations were much greater in barley than in wheat (*8*). In a similar study, Abramson et al. (*5*) subjected hulless barley to storage at 15% and 19% IMC for 20 weeks. At 20 weeks the OTA concentration of 19% IMC barley was 24 ng/g and in 15% IMC barley it was not detected.

Grain drying practices need to be efficient and effective in order to inhibit OTA producing fungi from becoming established, ultimately preventing post-harvest OTA contamination (*34*). The dissemination of safe storage practices remains a challenge in some countries that lack the infrastructure (i.e. funding, expertise) to advise and ensure that these practices are communicated to local farmers and involved parties (*176*).

Transport. Although the transport step may be relatively brief compared to storage, it should not be discounted. Containers and vehicles used to transport grain should be cleaned before and after use with periodic disinfection. Additionally, transportation vesicles should be pest-free. Conditions that could lead to sweating of the grain or moisture build-up should be avoided (*69*). In all post-harvest steps, freshly dried grain should be segregated from old and moist grain as well as grain dust (*75*).

Trucksess et al. (*191*) examined barley and winter wheat sampled from rail cars and trucks using a method with a LOD of 0.03 ng/g. The group reported that 36 samples of winter wheat (9.4%) and 11 samples of barley (10.7%) were OTA-positive at levels ranging from 0.03-31.4 ng/g and 0.1-17.0 ng/g, respectively.

Between October 1997 and June 1998, a total of 306 stored wheat (n=148), barley (n=131) and oats (n=21) samples were collected from trucks and elevators in the United Kingdom and analyzed for OTA (detection limit=0.1 ng/g). Oats had the highest incidence of OTA (28.6%) followed by barley (26.7%) and wheat (14.9%). The mean OTA concentration was 0.53, 2.60, and 1.94 ng/g, respectively. In general, the percentage of positive samples and the mean OTA concentration increased with storage time and moisture content (*179*).

Effect of Fungal Invasion on Cereal Grains

High levels of microbial activity have a direct deleterious effect on the quality and nutrition in grain (*127*). Such effects can limit the use of affected grain for animal feed, seed, or processing (*120*). In addition to mycotoxin production, fungi can produce volatile metabolites leading to off-odors, and can cause respiratory disease to exposed workers (*110, 122*). Chelkowski and Cierniewska (*39*) observed that protein in the aleurone layer of wheat and barley kernels were compromised as a result of mycelial invasion. Fungal invasion of corn resulted in a reduction of germination rate and carbohydrate, protein, and total oil degradation. An increase in moisture and free fatty acid content also resulted (*24*).

Effect of Processing on OTA

As was covered, OTA contamination can occur at the pre- harvest level but is more likely to occur after harvest during storage. The processing step can act to concentrate or decrease OTA levels in food. Such processes for cereal grains include sorting, cleaning, milling, brewing,

cooking, baking, and extrusion (*30*). OTA contamination can occur during types of processing

that provides conditions conducive to mold growth, such as fermentation, germination, and

malting (*23, 127*). On the other hand, OTA can breakdown when subjected to acidic or alkaline

conditions, high temperature, or enzymes (*176*). Other processes either have little to no effect on

OTA production or elimination, whereas some processes do achieve a level of OTA degradation.

One of the inherent challenges of OTA is that it is a very stable compound, having been reported

to remain at the same level during grain storage for over one year (*189*). Similarly, OTA also

does not readily degrade during processing (*96*).

Sorting is an effective way to remove damaged kernels and visible signs of fungal

contamination. However, this intervention is selective and does not break down the compound or

eliminate the organism (*30, 145*). Experiments conducted by Chelkowski et al. (*42*) showed that

both dry and wet cleaning of wheat and barley grain did not completely remove OTA.

Scudamore et al. (*178*) achieved only 2-3% reduction in OTA in barley during the cleaning

process.

Dry milling itself has little to no effect on OTA level, besides performing a level of

dilution or concentration depending on the mill fraction (*30*). OTA tends to be most concentrated

in the bran layer of cereals (*145*). Scudamore (*176*) reports OTA is concentrated 3-fold upon

milling. Correspondingly, removing only 1-2% of the surface layers (by weight) reduced OTA

level by 25-40%.

Chelkowski et al. (*42*) reported that approximately 10-50% of OTA was found on the

surface of the kernel. This conclusion was supported by the fact that approximately the same

amount of OTA was detected in both the bran and flour portions of the grains after milling. The

presence of OTA in the flour is attributable to the fact that ochratoxigenic fungi penetrate the

kernel, often reaching the endosperm. The process of pearling barley removed 70-90% of OTA from contaminated grain. This was in large part due to the removal of the hull (*42*).

In support of the aforementioned results, Osborne et al. (*145*) demonstrated that the cleaning process of physically scouring grain, which acts to remove part of the bran coat, prior to milling reduced OTA by more than 50% in both whole and white wheat flour. Scudamore et al. (*178*) reported a 25% reduction in OTA by the same process. White flour contains lower OTA concentrations than whole meal flour due to the removal of the bran and offal. Although the water activity of flour negates fungal growth and mycotoxin production, conidia present in the flour can survive for extended periods of time. Thus it is necessary that flour be properly stored and kept dry in order to prevent post-process OTA contamination (*32*).

During the brewing process OTA in grain can carry over to the finished product as OTA remains relatively stable throughout the process (*44*). Both the germination and malting steps are conducive to ochratoxigenic fungi growth, if present (*127*). Chu et al. (*44*) spiked OTA onto material at five different stages in the brewing process. OTA level was determined at the end of each step. OTA was reduced by 12-27% in malt mash, 20-30% in boiled wort, and another 20-30% during the final fermentation. A different study demonstrated that OTA increased 2-4-fold during malting in 75% of samples tested. The increase was related to temperature in that an increase in temperature resulted in an increase in OTA concentration (*176*). Baxter et al. (*16*) reported 40% loss in OTA during mashing and another 16% was removed in the spent grains. The final beer product retained 13-32% of the original spiked OTA. Scudamore (*174*) reported that 20% of original OTA in malt remained in the final product. Scott et al. (*175*) found that an 8-day fermentation by three different *Saccharomyces cerevisiae* strains resulted in a 2-13%

reduction of OTA, with some of that reduction being attributable to uptake by the fermenting yeast.

Dough fermentation and baking processes have little to no effect, respectively, on reducing OTA content (*127, 178*). Dough fermentation used in Spanish bread making significantly reduced OTA concentration by 29.8-33.5% (*193*). Duarte et al. (*54*) noted that the level of reduction achieved during fermentation may be dependent on the yeast strain used. In biscuit baking and breakfast cereal production OTA was reduced by ≥66%. Observed reductions were attributed to high temperatures involved in processing as well as lower final moisture content, as compared to bread making (*30, 188*). Cake baking did reduce OTA by 56%, presumably due to the higher moisture content of the product during heat treatment. OTA was reduced by 86% in biscuits upon baking whereas there was a 55% reduction in white bread. This difference was attributed to the higher dough temperature involved in making biscuits and a lower final moisture content (*144*).

During whole wheat-based breakfast cereal production, higher temperatures and moistures during extrusion resulted in greater decreases in OTA (*177*). Longer residence time also affected OTA levels, with the maximum loss being 40% (*30*). Scudamore (*176*) stated that most commercial processes do not exceed 180°C and therefore, in reality, OTA reduction is limited to 25%, depending on pH conditions. Similarly, in a study conducted by Castells et al. (*38*) artificially contaminated barley meal was extruded at a number of temperature, MC, and extrusion processing conditions. Optimal moisture for OTA reduction was 24-30%. More OTA was reduced as residence time increased, due to longer subjection to high temperatures, shear and high pressure. The greatest OTA reduction (86%) was observed at 160°C, 30% MC, and a

70 s residence time (*38*). Autoclaving dry oatmeal or rice cereal resulted in 86-87.5% loss of OTA (*30*).

It has been concluded by Pitt et al. (*159*) that, at the point of processing, routine OTA analysis and the rejection of lots that do not meet standard specifications is currently the only effective means by which to reduce OTA in human food and animal feed.

OTA Detoxification of Grain

OTA detoxification methods can be grouped into three broad categories based on the nature of the method: physical, chemical, or microbiological (*10*). Various physical methods by way of processing were discussed above. In addition, multiple methods have been effective in reducing conidia viability and thus preventing OTA production. Such methods include applying a freeze and thaw (-20/26°C) process, UV B, and gamma treatments (2-5 kGy). Both freeze/thaw and UV B have only been tested in liquid media. Only gamma irradiation has proven to destroy OTA once it has already been produced on grain (*13, 51*).

Due to the inability of physical processing to fully remove or break down OTA, a variety of chemical- and microbiological-based treatments have been investigated that aim to detoxify cereal grains by modifying or absorbing OTA. A 2% ammonia treatment of grain artificially contaminated with OTA was successfully broken down into a less toxic form within 4-6 weeks. The temperature (>15°C) and moisture level (>15%) of the grain was critical to the decomposition process. The authors note that this process is ideal for typical contamination levels seen in grain (0.5-4 ng/g) but highly contaminated grain (>50 ng/g) cannot be detoxified with this method. Nutritional losses were observed as a result of ammonia treatment, which would affect the value of treated grain intended for feed purposes (*41*).

Ozone in combination with electrochemical techniques has also been examined in reducing OTA. Aqueous OTA was exposed to various ozone concentrations over time. Results showed that OTA was completely destroyed within 15 seconds. Although this technology would result in only minimal nutrient loss in grain its efficacy has yet to be tested on infected grain (*129*).

Various fungal enzymes (e.g. carboxypeptidase A, lipase) have shown the ability to degrade OTA in liquid media by cleaving the compound (*51*). Other studies have investigated OTA degradation by bacteria (e.g *Streptococcus*, lactic acid bacteria, *Bacillus*), and fungi (e.g. *Aspergillus, Alternaria, Botrytis, Cladosporium, Penicillum, Rhizopus, Saccharomyces*) (*157, 196, 197*). Several *Aspergillus* strains were also able to entirely degrade OTA in liquid medium, with one *A. niger* isolate having efficacy in solid media as well (*197*). *Phaffia rhodozyma* degraded 90% of the OTA within 15 days in liquid broth (*157*). Lactic acid bacteria were able to degrade OTA by 80-95% in liquid medium but results were less pronounced (39-59%) when tested with tissue culture *in vivo* (*76*).

Varga et al (*196*) proposed the use of the post-harvest plant pathogen *Rhizopus stolonifer* to act as a biocontrol organism as isolates have demonstrated the ability to degrade OTA (>95% OTA within 16 days), as well as other mycotoxins, in liquid media and on moistened wheat. The authors did not address the fact that the actual application of such a prevention measure has not been tested in the field and they did not pose any associated caveats. Put simply, *Rhizopus* species are extremely fastidious and so growth would be difficult to control, let alone eliminate from the environment, if so desired. The effect that the organism might have on grain quality also requires further inquiry.

OTA Regulation in Food

To date over 99 countries around the world regulate OTA in an effort to protect consumers' health and ensure fair trade practices (*97, 195, 199*). Regulatory levels range from 0.5-50 ng/g, depending on the commodity or food product (*190, 199*). It is estimated that Europeans and Canadians are exposed to 15-60 ng OTA/kg body weight each week (*190*). Young children are the most at risk to OTA exposure because of their lower body weight (*89*). Based on toxicological data it was determined that the current maximum tolerable daily intake (TDI) of OTA for humans should be in the range of 1.2 to 14 ng/kg body weight (*66*). It is necessary to keep OTA exposure levels low due to the long half-life of OTA and the risk of bioaccumulation (*146*). In 2006 the European Food Safety Authority (EFSA) set the tolerable weekly intake (TWI) for OTA at 120 ng/kg body weight (*66*).

Although it is widely viewed that the risk of adverse health effects due to OTA is low for the majority of the population, it is recognized that measures should be taken to reduce exposure. The European community was the first to impose OTA regulations. In 1995 Denmark introduced maximum limits for OTA in grain and flour. In 2001 the same limits were adopted in the EU (*61*). Currently the EU has set maximum OTA levels ranging from 2-10 ng/g OTA in a variety of foods that are intended for direct human consumption (*97*). The maximum limit of OTA in grain-based foods for countries in the European Union, as established by the European Commission, is 5.0 ng/g in raw cereal grains, 3.0 ng/g in cereals intended for direct human consumption, and 0.5 ng/g in cereal-based baby food and formula (*64*). In 2009 the aforementioned maximum levels were proposed for Canadian food and beverages (*89*). To date, the United States does not regulate OTA in commodities, animal feed, or human food.

OTA Detection and Quantification

<u>**Sampling**</u>

In general, methods for determining OTA consist of lot sampling, sample preparation, extraction, clean-up, determination, and confirmation (*160*). The overall variability in testing is a function of sampling, sub-sampling, and analytical variability (*115*).Sampling is necessary for accurate results in any monitoring activity and is arguably the most essential step in OTA analysis (*190*). Thus the chosen sampling method can greatly affect the overall accuracy and precision of the entire method, especially in the case of granular food matrices, such as grains (*115*). Past studies involving mycotoxin testing have shown that sampling is the largest source of variance during detection, accounting for 25-88% of the total variance for the experimental conditions (*205, 206, 207*).

The general flow of the sampling analysis process for whole grains consists of five steps. Initially grain is divided into lots, which is defined as a unit that is delivered at the same time and is considered to have the same characteristics. A lot is divided into sublots and each sublot is sub-sampled to obtain incremental samples. Incremental samples are then combined, often by mixing, to form an aggregate sample. A sample intended for the laboratory is taken, which may consist of the entire aggregate sample or a pre-determined portion of the aggregate sample. The laboratory sample is ground and a sub-sample, or test portion, is taken for analysis (*190*). Representativeness of a sample, in terms of sampling procedures, is important when comparing published studies on OTA occurrence and levels, as well as year-to-year variation. Other factors to consider include changes in agricultural practices or processing methodologies (*102*).

Obtaining a representative sample is crucial; however, even upon implementation of the most robust plan a level of uncertainty remains. Homogenization occurs at some level with most

types of processing, which aids in decreasing variance and uncertainties. Sampling raw whole

grains is especially challenging due to the heterogeneous nature of the product and mycotoxin

production (Table 4) (*190*). Mixing aids in disrupting the localized spots or "hot spots" of mold

growth and OTA, evenly distributing them and thus attaining a more representative sample. In

the case of ground wheat Nowicki and Roscoe (*139*) found that 15-30 minutes of mechanical

mixing can significantly decrease the sampling variance. Sampling variance can also be reduced

by increasing the number of samples or the amount of grain per sample taken from the original

lot. Garcia-Fonseca et al. (*78*) demonstrated that a smaller particle size results in a greater

reduction of the relative standard deviation (RSD). In their study wheat ground to <1 mm gave a

RSD of 96%, particle sizes between 50-250 µm had 34% RSD, and particles <50 µm in size had

3% RSD.

Table 1.4. Ochratoxin A (OTA) concentrations detected in single kernels of Canadian western amber durum wheat inoculated with *P. verrucosum* and incubated in plastic bags.

OTA (ng/g)	# Kernels
<20	421
20-100	5
100-1000	3
1000-2000	7
>2000	4

Note: LOQ=20 ng/g
Adapted from Tittlemier et al., 2011.

A sampling study conducted by Biselli et al. (*27*) of a truck hauling wheat established

that, geospatially, OTA is highly variable. In the study 100 incremental samples were taken

following a grid-like sampling plan that adhered to the official European Commission Regulation

401/2006 (*66*). The regulation recommends that for 300-1500 ton lots, the lot should be divided

into 3 sub-lots and a total of 100 incremental samples taken from each sub-lot and combined to

form an aggregate sample size of 10 kg. In this case samples were analyzed individually as well

as in aggregate. OTA was detected in localized areas or "hot spots" in which concentrations ranged from <0.2-8.6 ng/g OTA. The averaged OTA concentration based on the incremental samples was 0.6 ng/g whereas the aggregated sample value showed "no coherence" to the aforementioned value. Consequently, extensive sample comminution of the entire volume of grain under question is imperative to attain a representative sample (*27*).

The effects of dry milling (grinding followed by 60 min of mechanical and hand mixing) and slurry mixing (grinding, water added prior to mechanical mixing) on OTA determination in wheat were studied. Both samples were purified using an OchraTest™ immunoaffinity column. The LOD was 0.1 ng/g. Only slurry mixing provided high accuracy and low variability (99.4% recovery, CV 10%) whereas dry milling resulted in a non-homogenous distribution of OTA (43.2% recovery, CV 110%). This study demonstrated that variability increased as the level of OTA in a sample decreased. Slurry mixing is crucial in order to obtain accurate results in samples containing low OTA levels (*115*).

In the case of countries with OTA regulations, sampling for routine monitoring of OTA in grain presents challenges to companies as sampling can be an expensive and time-consuming operation. International Organization for Standardization (ISO) has a standard for sampling of cereals both under static and dynamic conditions. The ISO states that sampling product while it is moving obtains a more representative sample than static sampling (*100*). This is supported by a study conducted by Andersson et al. (*12*) in which they compared automatic and manual sampling from a moving stream of barley that had been spiked with *P. verrucosum* and subsequently incubated to allow for OTA production to occur. Results of the study showed automatic sampling had significantly lower uncertainty than with manual sampling. Thus it was

recommended that an automatic sampler placed after a mixer offers an accurate and cost-effective way of gaining representative samples for OTA analysis (*12*).

To date no correlation has been made between the presence or infection/infestation rate of *P. verrucosum*, OTA production, and kernel size (*190*). However, it is known that OTA is more prevalent in the bran and chaff. Thus OTA becomes unevenly distributed in grain fractions after milling. Therefore special attention must be paid to stratification when sampling in order to minimize error and reduce uncertainty (Table 5). One way to do this is to use a sampler that has an opening that is 2-3 times the size of the largest kernel (*190*). Biselli et al. (*27*) recommends that grain samples are coarsely ground first, followed by a thorough mixing step prior to grinding to the final particle size.

Table 1.5. Distribution of ochratoxin A (OTA) in fractions of coarsely ground barley after sieving.

Particle Size (mm)	Fraction Mass (g)	Fraction mass (%)	OTA (ng/g)	SD	OTA (ng/fraction)	OTA (%)
>2	78	3.4	0.4	0.1	31	0.1
>1	1026	45.2	4.9	0.2	5027	14.4
>0.5	682	30.0	9.7	1.4	6615	19.0
>0.25	244	10.7	32.5	2.1	7930	22.7
<0.25	240	10.6	63.7	1.7	15,288	43.8
Aggregate	2270	100.0	15.4	--	34,892	100.0

Adapted from Andersson et al., 2011.

The Canadian Grain Inspection Commission (CGC) and the United States' Grain Inspection, Packers and Stockyards Administration (GIPSA) provide recommendations for sampling for grading purposes. The procedures consist largely of a variety of probing patterns for stationary lots of grain (*36, 85*).

After a sample has been obtained and prepared for analysis (if necessary), the sample has to undergo extraction, clean-up, determination, and confirmation. There is an array of options for achieving OTA detection and/or quantification, each with particular advantages and

disadvantages. Although steps subsequent to sampling are less prone to error, variations in food matrices present another problem. Currently, no "gold standard" method exists for any specific cereal grain. The method one selects will be dependent on the desired outcome as well as other factors such as time, cost, and technical expertise.

Detection and Quantification

Thin-Layer Chromatography (TLC). Given the fluorescent properties of OTA, reference methods have been based on chromatography (*88*). The majority of the official methods validated for detecting OTA in foods involves the use of TLC or liquid chromatography with fluorescence detection (LC-FLD) with either an immunoaffinity column (IAC) or solid phase extraction (SPE) (*201*). Such methods are accurate and reproducible and results are produced in hours. However, personnel must be skilled and procedures can be costly (*88*).

The official AOAC method for barley (LOD = 12 ng/g; COV = 31-54%) is based on TLC (*136*). OTA and OTB are extracted from ground barley samples after acidification using chloroform. The toxins are retained in the column containing diatomaceous earth and basic aqueous solution. After clean-up, the ochratoxins are eluted and TLC is performed using UV irradiation to visualize fluorescent ochratoxin spots (*210*).

High-Performance Liquid Chromatography (HPLC). HPLC is a sensitive method by which to detect and quantify OTA in grains. OTA is soluble in polar organic solvents and dilute aqueous bicarbonate and slightly soluble in water. This property was leveraged to extract OTA in a variety of methods (*210*). In grain, extraction generally consists of chloroform and an acid and then partition using sodium bicarbonate (*142*).

HPLC methods consist of extraction in acetonitrile/water or chloroform/phosphoric acid. Purification, or clean-up, is achieved using immunoaffinity columns (IAC), liquid-liquid

extraction, or solid phase extraction (*96*). In the AOAC method the extract is cleaned using a

C18 column and OTA eluted using ethyl acetate-methanol-acetic acid (*142*). Currently, OTA

analysis is almost exclusively done with HPLC and various detectors, such as ultraviolet,

fluorescence, or mass spectrometry. Derivatization to verify detected OTA is performed in the

last step. In the AOAC method, OTA is identified using reversed-phase liquid chromatography

and quantified using fluorescence. The presence of OTA is confirmed by detection of the methyl

ester derivative (*142*). The OTA methods adopted as "European Standards" are based on HPLC,

mostly in combination with IAC (*182*).

Aboul-Enein et al. (*4*) published a modified HPLC method for the detection of OTA by

HPLC with fluorescence detection in wheat, corn, red pepper, cheese, and wine. They proposed

two different extraction procedures. The first involved a chloroform extraction and dissolving the

final residue into ethanol prior to injection into the HPLC. The second was an ethanol extraction.

The LOD and LOQ were 0.1 ng/g and 3.3 ng/g, respectively. The combined average recovery

for all matrices was 81.2±1.9%. The authors do not state LOD, LOQ, or recovery values for

individual matrices tested or how each matrix was prepared prior to extraction.

HPLC-FLD is considered highly selective and sensitive. Although HPLC paired with

mass spectrometry (HPLC-MS) is as well, the sample matrix can cause issues whereas it is an

insignificant issue in HPLC-FLD (*96*). The use of ultra-high performance liquid chromatography

(UHPLC) has been explored to simultaneously detect multiple mycotoxins. UHPLC is even

more sensitive and specific than HPLC-FD and HPLC-MS. Additionally, the use of a low-

volume column and high column temperature reduces analysis time and solvent use. While

comparing methods Ibáñez-Vea et al. (*96*) used acetonitrile and water for extraction of all

targeted mycotoxins; however, greater recovery rates were obtained for OTA in methanol and

ethanol. In barley, the LOD and LOQ were 0.01 ng/g and 0.15 ng/g, respectively. Recovery rates ranged from 76.7-89.3%.

When it is preferable to detect and quantify multiple mycotoxins in a sample liquid chromatography-mass spectrometry (LC-MS) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) can be used in order to decrease the total amount of sample and reagents required and analysis time. Soleimany et al. (*183*) used LC-MS/MS for simultaneously detecting 4 aflatoxins, ochratoxin A, zearalenone, deoxynivolenol, 2 fumonisins and the T2- and HT-2 toxins in cereals. LC-electrospray ionization (ESI)-MS/MS has been used to detect OTA down to a level of 0.02 ng/g in pig kidneys (*201*).

Immunoassays. It is generally accepted that laboratory-based methods such as HPLC provides the most accurate results but not all situations can utilize that method. Some applications require routine analysis of samples with results within minutes to hours in the absence of a sophisticated laboratory or skilled personnel (*182*). Immunoassays have the potential to fill this need. Immunoassays are based on the ability of an antibody to specifically bind select target molecules based on the physical structure of the target (*88*).

Monoclonal and polyclonal antibodies specific for OTA have been developed. Enzyme-linked immunosorbent assays (ELISA) have antibodies or antigen that are immobilized on the bottom of microtiter plates, which are then subjected to a competitive process. Although several types of ELISA formats exist, direct competitive ELISA is most commonly used for OTA applications. Ochratoxin A and an ochratoxin-enzyme conjugate compete for the binding sites of anti-OTA antibodies which are immobilized on the wells of the microtiter plate. After a washing step and addition of the substrate solution, a color signal is produced. After a pre-specified time

the color reaction is stopped and the absorbance is read. The OTA concentration of a sample is determined by interpolation using a standard curve (*20*).

Recently, an ELISA was developed for use of OTA detection and quantification in cereals. The limit of detection was 0.15 ng/mL (*214, 215*). In addition, several commercial test kits for OTA detection are available. Detection levels are typically in the 0.25-40 ng/g range, although each kit varies (*9, 25, 57, 135*). Not all kits are suitable for all matrices.

The advantages of ELISA are ease of use, portability, and that the test requires only a small sample volume (*88*). A level of sample preparation is required, especially for solid samples but ELISA does not require clean-up and is relatively inexpensive. Disadvantages include false-positives resulting from cross-reactivity or false-negatives when ELISA antibodies are inhibited by matrix components. The latter issue is not necessarily one that can be attributed to any one kit manufacturer as false-positives can occur between samples with the same matrix when using the same kit (*182*). Additionally, this method has only been validated for certain matrices and can be inaccurate when OTA is present at low concentrations (*153*).

Lateral flow device, or the immunochromatographic test strip, is an immunoassay made up of a membrane strip with antibody-coated receptors that bind to the target analyte. A liquid sample is applied to the device and the sample migrates via capillary action along the strip. Once the target is bound a color develops (*153*). The advantages and disadvantages of this technology are similar to that of ELISA. Unlike ELISA, this test is used for qualitative or semi-quantitative purposes only. Laura et al. (*112*) describes the development of a lateral flow assay for detection of OTA in maize and wheat. The reported detection limit of different test strips developed for cereal samples ranged from 1-500 ng/g (*43, 111, 204*).

Although immunoassays are portable, easy to use, and require minimal reagents, the test is only suitable for single use and presumptive positive samples require confirmation using an approved method, such as liquid chromatography (*201*).

Microbiological media. Culture media are relatively inexpensive but requires more time than rapid test kits. Also, this method only provides presence or absence testing (*201*). Nonetheless, a single plate can provide multiple layers of information that differs from the previously mentioned methods including, but not limited to, the selective or non-selective analysis of viable fungi as well as the presence of mycotoxins.

Coconut cream agar (CCA) was originally developed to detect aflatoxin production by *Aspergillus* species (*55*). This method involved the point-inoculation of isolates onto CCA and incubating for 3-7 days. After incubation the medium was examined for fluorescence using UV light. The presence of fluorescence confirmed the presence of aflatoxin. To date, the chemical basis for this fluorescence has not been determined (*55*). Several other investigators have since studied the use of this medum in detecting OTA production. Heenan et al. (*90*) determined the medium's applicability for detecting OTA from *Penicillium* species, *A. carbonarius* and *A. niger* isolates (N=148) using CCA and comparing results to those obtained using TLC. In both techniques OTA is detected using long wave UV light. The CCA method detected OTA production by 91% of the isolates compared to 82% by the TLC method. It was concluded that CCA is a valid, sensitive qualitative method for OTA detection (*90*).

Yeast extract sucrose (YES) agar and Czapek yeast agar (CYA) have also been used to screen *Aspergillus* isolates for OTA production (*29, 90*). The authors took agar plugs of the isolates, performed an extraction, and then ran the extract on HPLC to quantify the level of OTA (*29*). No statistically significant differences were found in the amount of OTA recovered using

YES and CYA. Extraction solvents methanol and methanol/formic acid (25:1) gave the best recovery of OTA.

Analytical methods for detecting and quantifying OTA have improved remarkably with the advent of immunoaffinity columns and high performance liquid chromatography (*102*). This increase in sensitivity should be kept in mind when making comparisons in OTA frequencies between years. Until a standard method for any one type of commodity or food is recognized and employed worldwide, OTA detection and quantification are inherently variable, and can only provide a snapshot of contamination levels in a given commodity (*54*).

Detection and Quantification of Ochratoxigenic Fungi

<u>Traditional Techniques</u>

Routine detection of ochratoxigenic molds has been conducted by plating a sample directly or by performing serial dilutions of a ground sample and plating onto a selective microbiological medium. Incubation is typically conducted over 7-15 days and then results read (*88*). Media that have been used to detect ochratoxigenic fungi include dichloran rose-bengal chloramphenicol (DRBC) agar, dichloran 18% glycerol (DG18) agar, or dichloran rose bengal yeast extract sucrose (DRYES) agar (*92, 105*). More recently DYSG (dichloran yeast extract sucrose 18% glycerol) was developed and has since proven significantly better at *P. verrusocum* detection than the aforementioned media on multiple levels (*117*).

On DYSG 98% of *P. verrucosum* isolates tested (N=86) produced a red-brown to terracotta brown reverse color which has been identified as an unknown anthraquinone by Frisvad et al. (*75*). This color allows for easy differentiation of *P. verrucosum* from other *Penicillium*. The colony reverse of *P. verrucosum* is light cream to light pink with no green sporulation (*117*). DYSG also selects against fastidious fungi including *Rhizopus* and *Mucor*

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species and reduces *Eurotium* sp. growth (*73, 117*). In the case of *Aspergillus sp.* Lund and Frisvad (*117*) reported *Aspergillus* growth in 2/19 (10.5%) samples contaminated with ≥5 ng/g OTA that had been plated onto DYSG. In addition, secondary metabolites are produced on DYSG in large amounts, which allows for direct confirmation of OTA production without the need for subsequent culturing (*73*).

Czaban and Wróbleska (*49*) leveraged the ability of DYSG to estimate the abundance of *P. verrucosum* in substrates containing mixed fungal populations. The group reported that *P. verrucosum* colony diameter surrounding wheat kernels directly plated on DYSG correlated with the CFU count. A level of 10^2 CFU/g and greater of wheat resulted in 100% *P. verrucosum* infestation. When wheat kernels were inoculated at ≤100 CFU/g, no *P. verrucosum* colonies were observed. Elmholt et al. (*60*) found that DYSG is a useful medium in estimating *P. verrucosum* levels in soil samples at levels as low as 200 CFU/g.

Limitations of these methods are that they are laborious, time consuming, and depending on the chosen growth medium, personnel may need to be highly trained in order to properly identify isolates (*88, 138*).

Molecular Techniques

DNA-based techniques are a good alternative to traditional culture techniques. Advantages include sensitivity, specificity, accuracy, and results can be obtained within a day (*82*). However, sample preparation and DNA extraction can increase total analysis time to several days. Although culture- and molecular-based techniques both require trained personnel, DNA-based techniques remove the necessity for high level expertise required for fungal identification and subjectivity inherent to that task. One major limitation is that results are

nondiscriminatory between viable and non-viable fungi and are ultimately dependent on the quality of the primer design.

Fungal isolates have been identified to the species level using one or both of the intergenic transcribed spacer (ITS) regions of the ribosomal operon as targets in traditional polymerase chaing reaction (PCR) assays in order to discriminate between ochratoxigenic *Aspergillus* species (*82, 154*). The first ITS sequence is located between the 18S and 5.8S rRNA genes and the second between 5.8S and 28S rRNA genes. After transcription both ITS regions are excised and therefore these regions accumulate more mutations than more conserved sequences that are related to specific cellular functions (*138*). This inherent variability allows for a high level of discrimination between and within species (*88*). The limitation of this approach is that one cannot differentiate OTA producers from non-producers.

Others have leveraged differences in genes known to be involved in the OTA biosynthetic pathway in order to detect and differentiate ochratoxin producers. The polyketide synthase gene (*pks*) is believed to only be present in ochratoxigenic fungi (*88*). Multiple groups have developed PCR methods targeting the OTA polyketide synthase gene (*otapks*PN) and non-ribosomal peptide syntethase gene (*otanps*PN) from *P. nordicum* (*28, 118*). *P. nordicum* gives a positive result for both genes whereas *P. verrucosum* is only positive for *otanps*PN. Ochratoxigenic Aspergillus species are also positive for *otanps*PN (*164*).

Real-time or quantitative PCR (qPCR) retains the sensitivity of PCR but allows for quantification of the PCR product during the reaction. Amplification of the target is detected either by a fluorescent dye such as SYBR Green that binds non-discriminately to any double-stranded DNA or by labeled TaqMan probes. SYBR Green is lower in cost compared to probes but TaqMan allows for greater specificity given that fluorescence takes place only when the

specific targeted sequence is annealed. In wheat, methods based on both SYBR Green and TaqMan have been developed for detection and quantification of *P. verrucosum* based on the sequence of the OTA polyketide synthase gene, *otapks*PV, as well as *P. nordicum* (*otapks*PN) (*80, 174*). Rodríguez et al. (*165*) designed SYBR Green primers and TaqMan probes targeting *otanps*PN in order to quantify ochratoxigenic *Aspergillus* and *Penicillium*. The LOD varied from 1-10 conidia/g in artificially inoculated cooked turkey breast, cooked ham, mortadella, dry-cured ham, dry-fermented sausage, ripened cheese, grape, plum, and pear. In a different study, ochratoxigenic strains belonging to the *A. niger* aggregate were targeted by way of a polyketide synthase (*otapks*AN) located in a gene cluster that is putatively involved in OTA biosynthesis using both TaqMan and SYBR Green protocols. The method was not tested using naturally contaminated foods or commodities (*37*).

Multiplex PCR involves the simultaneous detection of multiple PCR targets in a single test by the use of more than one primer set. This approach saves time and reagents. The challenge is designing sets of primers that are rigorous at a common annealing temperature and elongation times (*88*). A multiplex PCR method using TaqMan probes was developed for detecting and quantifying aflatoxins-, OTA-, and patulin-producing fungi in a single food sample. A variety of artificially-inoculated food matrices were tested including: wheat, peanuts, paprika, grape, apple, peach, pepper, turmeric, oregano, dry-cured ham, dry-cured sausage, and dry-ripened cheese. The LOD was 10^1-10^3 CFU/g, for aflatoxin and patulin producers, whereas it was 10^1 CFU/g for OTA producers. Naturally contaminated samples were not included in the study (*164*).

The trend toward sustainability increases the desirability of a procedure that uses little to no organic solvents. In addition, OTA detection methods are being designed in order to increase

sensitivity while decreasing cost and labor involved. An example of such a technology is capillary zone electrophoresis with laser induced fluorescence (CZE-LIF). Advantages of CZE-LIF over more traditional methods such as LC, along with the fact that it uses small sample volumes, is less expensive and does not require organic solvents, is that it is more sensitive and gives better separation from interfering compounds (*201*).

Research Objectives

The collective knowledge surrounding the effects of human exposure to OTA has greatly advanced since its discovery in 1965. However, much remains to be discovered. In particular, data regarding the prevalence of OTA on commodities here in the United States is lacking. Such information will be a key component in building the case as to whether or not there is a need to regulate this toxin domestically. This project aims to address OTA prevalence in barley and wheat grown in the northwestern and Great Plains regions of the United States as well as other relevant research questions as described in the following objectives:

Objective 1 – OTA Prevalence and Level in Freshly Harvested Barly and Wheat

It is widely accepted that OTA in grain is an issue that arises when grain is subjected to improper or sub-optimal storage conditions. It is also known that ochratoxigenic fungi are present on the grain in the field and during harvest. Therefore it is possible that OTA could be produced prior to storage. We hypothesize that OTA is present in grain directly after harvest. This objective aims to determine the prevalence and level of OTA in freshly harvested barley, durum wheat, and hard red spring wheat.

Objective 2 – OTA Prevalence and Level in Stored Barley and Wheat

Due to the nature of commodities, wheat and barley are typically stored for a length of time before being processed into food or feed. This objective aims to determine the prevalence

and level of OTA in stored barley, durum wheat, and hard red spring wheat. It is hypothesized that OTA becomes more prevalent and is present at greater concentrations the longer a sample is stored.

Objective 3 – Correlation Between Infestation, OTA Level, and *otanps*PN

Whether OTA is detectable in a grain sample or not, the presence of OTA-producing fungi suggests the potential for OTA production and contamination of the grain given environmental conditions favorable to fungal growth. This objective involves using real-time PCR to quantify *otanps*PN, a gene involved in OTA biosynthesis, of OTA-producing molds in composited samples of freshly harvested and stored barley, durum and hard red spring wheat with undetectable levels of OTA and OTA-positive samples (>1 ng/g). We hypothesize that OTA level and ochratoxigenic fungi biomass are correlated in that samples with a higher OTA concentration will also have a greater number of *otanps*PN.

Supporting objectives include isolating possible OTA-producing fungi and performing identification to the species level using a traditional plating technique. We suspect that *Penicillium verrucosum* is the primary OTA producer in these samples. Additionally, a representative number of presumptive OTA-producing isolates will undergo screening to confirm OTA production *in vitro.* We believe that not all presumptive OTA-producers will produce OTA *in vitro.*

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CHAPTER 2[1]. SURVEY OF OCHRATOXIN A IN FRESHLY HARVESTED BARLEY, DURUM AND HARD RED SPRING WHEAT IN THE UNITED STATES, 2011-2012.

Abstract

Ochratoxin A (OTA) is a toxin produced by some *Penicillium* and *Aspergillus* species in a variety of food and feed, especially in cereal grains, around the world prior to harvest but primarily during storage. Barley, durum and hard red spring (HRS) wheat samples were collected right after harvest as part of regional crop quality surveys in both 2011 (N=653) and 2012 (N=717) from the Upper Great Plains. Barley samples were collected independent of the regional survey in 2012. All samples were analyzed for OTA contamination using high-performance liquid chromatography (HPLC) with fluorescence detection (FLD). Overall, 2.7% of the samples were positive for OTA. In 2011, OTA was detected in 1.0% of the durum wheat samples but was not found in HRS wheat or barley. In 2012, 19.0%, 8.3%, and 1.4% of the barley, durum and HRS wheat samples, respectively, were positive for OTA. Of the 37 samples that had detectable levels of OTA, 3 samples (12%), all of which were durum wheat, exceeded 5 ng/g OTA.

Introduction

Mycotoxins are secondary metabolites produced largely by some *Aspergillus*, *Fusarium*, and *Penicillium* species under sub-optimal storage conditions (*12*). Ochratoxin A (OTA) is the most prevalent and potent of the ochratoxins. It has been reported as an immunosuppressant, a possible human carcinogen, as well as toxic to several organs including the kidney, liver, and nerves (*20*). The genera and species that produce OTA depend largely on the climate of the

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region and the substrate. In cold and temperate climates, such as Europe, Canada and the United States, *Penicillium* species are the most common OTA producers. *P. verrucosum* are the source of OTA in cereals and cereal products whereas *P. nordicum* has been isolated from cheese, and fermented or dried meats and fish. In tropical climates *Aspergillus* species, such as *A. ochraceus, A. niger, A. carbonarius, A. westerdijkiae,* and *A. steynii* predominate (*9, 15, 24*).

OTA has been found worldwide in animal feed and a variety of commodities and foods including oats, wheat, rye, barley, corn, fruits, coffee, spices, fruit juice, wine, beer, beans, pork, poultry, milk, and infant formula (*1, 7, 17, 21, 28, 29, 31, 32, 34*). Humans can ingest OTA either directly from foods tainted with OTA or indirectly by consuming meat or milk from animals fed with OTA-contaminated feed (*12*). According to the European Food Safety Authority and the Joint Committee FAO/WHO of Experts on Food Additives the current maximum tolerable daily intake for humans is estimated at 14 or 17.1 ng/kg of body weight, respectively, due in large part to the long half-life of OTA (*14, 16*). It has been reported that cereals and cereal-based products are the most significant daily source of OTA in the human diet (*28*). The maximum limit of OTA in foods for countries in the European Union, as established by the European Commission, is 5.0 ng/g in raw cereal grains, 3.0 ng/g for cereals intended for direct human consumption, and 0.5 ng/g for cereal-based baby food and formula (*13*).

Although multiple studies have been conducted in Europe and Canada examining the occurrence and concentration of OTA in multiple commodities and food products, data is lacking for the United States, where guidance levels and regulations do not exist. The aim of this study was to determine the prevalence and level of OTA in freshly harvested barley, durum and HRS wheat grown in the Northern Great Plains region of the United States.

Materials and Methods

Grain Collection and Samples

Samples were obtained in 2011 and 2012. Both years HRS wheat samples were collected during harvest by offices of the Minnesota, Montana, North Dakota and South Dakota National Agricultural Statistics Service (*5, 6*). Durum wheat was collected by the Montana and North Dakota National Agricultural Statistics Service offices (*3, 4*). In 2011 barley was gathered by offices of the North Dakota and Minnesota National Agricultural Statistics Service offices (*2*). All surveys were under the supervision of the Department of Plant Sciences at NDSU. Due to budget constraints in 2012, a regional barley sample surveywas not conducted by the Department of Plant Sciences. Barley samples were obtained from commercial sources, but the locations sampled were similar to those used in the 2011 survey.

Approximately 0.91-1.36 kg samples were collected throughout the harvest season with >80% of the samples coming directly from growers on the field and the remainder from farm bins and local elevators. Samples were stored in sealed moisture-proof plastic bags. Sample collection was weighted based on the projected production number (bushels) for each county. The 2011 regional wheat survey consisted of 103 durum and 457 HRS wheat samples. Sixteen of the HRS wheat samples were composites. A total of 93 barley samples were collected. In 2012, 63 barley, 217 durum and 437 HRS wheat samples were collected. In addition, 98 non-graded durum wheat samples were included in the 2012 analysis. These samples were collected as part of the regional survey but not graded.

Each sample was stirred to mix and 100 g sub-samples were collected and stored at -18°C until analysis. The average moisture content of the graded samples for 2011 and 2012 was 11.6%

and 10.5% (durum wheat), 11.8% and 11.6% (HRS wheat), and 13.3% (barley), respectively, as reported in the annual regional quality reports and by personal communication (*2, 3, 4, 5, 6*).

Ochratoxin A Determination

Samples were analyzed using AOAC method 991.44 (*27*) with a few minor alterations to the procedure (*22, 26*). Samples were milled (Perten Instruments, Model #3600, Hägersten, Sweden) to a fine powder, mixed, and stored frozen in sealed plastic bags until analyzed. Samples were defrosted and allowed to warm to 25°C prior to analysis. A 50 g portion of the ground sample was added to a flask, along with 25 mL 0.1M phosphoric acid, 250 mL chloroform, and then capped. The contents were shaken at ~175 rpm (25°C) for 80 minutes. Diatomaceous earth (~10 g) was added to the extract. The extract was filtered and each sample filtrate was transferred to two centrifuge tubes, 5 mL of 3% sodium bicarbonate was added to each tube, and then shaken to mix. The emulsion was centrifuged at 851 x *g* for 5 minutes and the upper phase was collected and frozen until final extraction. Samples were allowed to thaw while columns were prepared. Five milliliters of sodium bicarbonate extract was cleaned with a Strata C18-E column (Phenomenex, Inc., Torrance, CA). The column was then washed and OTA eluted. The upper phase was transferred to a secondary test tube and evaporated just to dryness under nitrogen. The residue was dissolved in 500 μl mobile phase, capped and vortexed. The solution was filtered using a 0.45 μm microfilter (Pall Corporation, Port Washington, NY) into a 1.5-mL HPLC vial. Confirmation of OTA was confirmed by preparing its methyl ester.

Samples were analyzed using a Shimadzu (Shimadzu Scientific Instruments, Columbia, MD) high-performance liquid chromatography (HPLC) system (LC-20A) that consisted of a gradient pump (LC-20AD), autosampler (SIL-20A HT) and fluorescence detector (RF-20A). The column (Phenomenex, Inc., Torrance, CA) was 250 mm x 4.6 mm (i.d.) packed with 5 μm

C18 bonded silica gel. The analysis was performed under isocratic conditions using a mobile phase of acetonitrile-water-acetic acid (51+47+2, v/v/v) at a flow rate of 1.0 ml/min. The fluorescence detector was set at an excitation wavelength of 333 nm and an emission wavelength of 464 nm. An injection volume of 25 μL was used and the retention time was approximately 10 min. The column oven (CTO-20A) was set at 40.0°C.

A standard solution of OTA (Sigma-Aldrich, St. Louis, MO, USA) was prepared in methanol according to the concentration established with a calibrated UV spectrophotometer. The standard was evaporated to dryness and then dissolved in mobile phase. A set of 5 standard solutions were prepared in the range of 0.2 to 2.1 ng and a standard curve was established daily. The calibration curve proved linear. The limit of detection (LOD) and limit of quantification (LOQ) for each grain were the concentrations of OTA that resulted in a signal-to-noise ratio of 3 and 10, respectively (*22*). For barley and HRS wheat, the LOD was 0.06 ng/g and the LOQ was 0.19 ng/g. The LOD was 0.09 ng/g and the LOQ was 0.30 ng/g for durum wheat. Recovery rates were determined by spiking known blank samples with multiple levels of OTA. Three replicates were performed each day on 3 separate days for a total of 9 samples per level per grain type. Spiked samples were left for 1 hour for the solvent to evaporate prior to extraction. Recovery rates were similar to that reported by Larsson and Möller (*22*) and Nesheim et al. (*26*). Confirmation of samples positive at levels >1.0 ng/g OTA was performed by methyl ester formation (*27*).

Statistical Analysis

The binomial proportions obtained for each grain type were tested for significance between 2011 and 2012 with the Chi-square test using SAS software (SAS Institute, Inc., Cary, NC, USA).

Results and Discussion

In total, 156 barley, 320 durum, and 894 HRS wheat samples were surveyed for OTA (Appendix A.1.-A.6.). In 2011 only 1 (1.0%) durum sample was positive for OTA. That same year none of the barley or HRS wheat samples had detectable levels of OTA (Table 2.1.). In 2012, the number of samples positive for OTA increased for all commodities. For durum wheat, 18 (8.3%) of the samples were positive and 6 (1.4%) HRS wheat samples were positive for OTA. In the case of barley, a total of 12 (19%) samples were positive. The binomial proportions were significant ($p<0.05$) for all three grain types between 2011 and 2012.

Table 2.1. Number (%) of barley, durum and hard red spring (HRS) wheat samples positive for OTA (ng/g) per year.

Year	Grain Type	No. Positive Samples/Total (%)	OTA (ng/g) Range	Median	Mean
2011	Barley	0/93 (0.0)	--	--	--
	Durum	1/103 (1.0)	5.56	5.56	5.56
	HRS	0/457 (0.0)	--	--	--
2012	Barley	12/63 (19.0)	0.15-0.90	0.26	0.38
	Durum	18/217 (8.3)	0.17-9.11	0.33	1.17
	HRS	6/437 (1.4)	0.21-1.97	0.55	0.86

OTA levels in the 12 positive barley samples ranged from 0.15-0.90 ng/g. The level of OTA in the 19 positive durum wheat and 6 positive HRS wheat samples ranged from 0.17-9.11 ng/g and 0.21-1.97 ng/g, respectively (Table 2.2.). The maximum limit of OTA in foods for countries in the European Union, as established by the European Commission, is 5.0 ng/g in raw cereal grains (*13*). Of the 19 positive durum wheat samples, 3 contained >5.0 ng/g OTA (5.56, 6.78 and 9.11 ng/g). None of the positive barley or HRS wheat samples exceeded 2 ng/g OTA.

In the past, two publications have utilized surveys to examine the occurrence of OTA in barley and/or wheat produced in the United States. The first was published by Shotwell et al. (*30*) in which 848 graded samples of hard red winter (HRW), hard red spring (HRS), and soft red

winter (SRW) wheat were analyzed between 1970 and 1973. OTA was detected in a total of 11 samples (1.3%). A total of 3 (1.0%) HRW wheat samples were positive ranging from <15-35 ng/g whereas the 8 (2.8%) positive samples of HRS wheat were higher at 15-115 ng/g. It should be noted that the detection limit of the method employed was >15 ng/g, a relatively low level of sensitivity compared to current methods.

Table 2.2. Range (ng/g) and mean (ng/g) of ochratoxin A (OTA) in positive barley, graded and non-graded durum wheat, and hard red spring (HRS) wheat.

| Grain Type	Number of Samples	OTA (ng/g)	
		Range	Mean
Barley	144	<0.06	<0.06
	2	0.06-0.20	0.15
	5	0.21-0.30	0.23
	0	0.31-0.40	--
	2	0.41-0.50	0.48
	1	0.51-0.60	0.59
	2	0.61-0.99	0.78
Durum	301	<0.09	<0.09
	5	0.09-0.20	0.18
	4	0.21-0.30	0.24
	1	0.31-0.40	0.37
	5	0.41-0.50	0.46
	1	0.51-0.60	0.57
	0	0.61-0.99	--
	3	1.0-10.0	7.15
HRS	888	<0.06	<0.06
	0	0.06-0.20	--
	2	0.21-0.30	0.26
	0	0.31-0.40	--
	1	0.41-0.50	0.49
	1	0.51-0.60	0.60
	0	0.61-0.99	--
	2	1.0-10.0	1.77

A more recent paper by Trucksess et al. (*33*) examined barley and winter wheat sampled from rail cars and trucks using a method with a LOD of 0.03 ng/g. The group reported that 36 samples of winter wheat (9.4%) and 11 samples of barley (10.7%) were OTA-positive at levels ranging from 0.03-31.4 ng/g and 0.1-17.0 ng/g, respectively. In 2009 and 2010, the Canadian

Food Inspection Agency tested 150 cereal grains for OTA, of which 75 were wheat products (i.e. flour, bran, couscous, and bulgur wheat). A total of 22 (29.3%) samples were positive for OTA, ranging from 0.3-2.5 ng/g. The LOD of the method was 0.1 ng/g (*8*).

The results of this survey showed that there were a significantly larger number of OTA-positive samples in 2012, as compared to 2011, for both wheat types. This may be due in part to the difference in weather conditions, namely temperature and precipitation, between these two years. As stated by the 2011 Durum Wheat Regional Quality Report (*3*), weather conditions consisted of "extremely wet conditions and flooding that delayed planting…" followed by wet conditions in the growing season (Figure 1). Weather during the harvest season was dry with "…above average temperatures." The 2012 crop year began with warmer than normal conditions, progressing to "… hot, dry conditions…" which continued to prevail throughout the end of the growing season and harvest (*4*). The aforementioned trends were also observed during the planting, growing, and harvest season for HRS and barley (Figures 2.2. and 2.3). As the harvest was characterized as hot and dry in both years, other factors such as the effect that weather conditions from a previous crop season may have on a successive season or the effect that planting and growing conditions have on OTA presence and concentration after harvest are worth consideration. In the past other groups have associated weather conditions with OTA prevalence.

Figure 2.1. Average* high temperature (A) and precipitation (B) per month during the durum wheat planting, growing, and harvest seasons in 2011 and 2012.
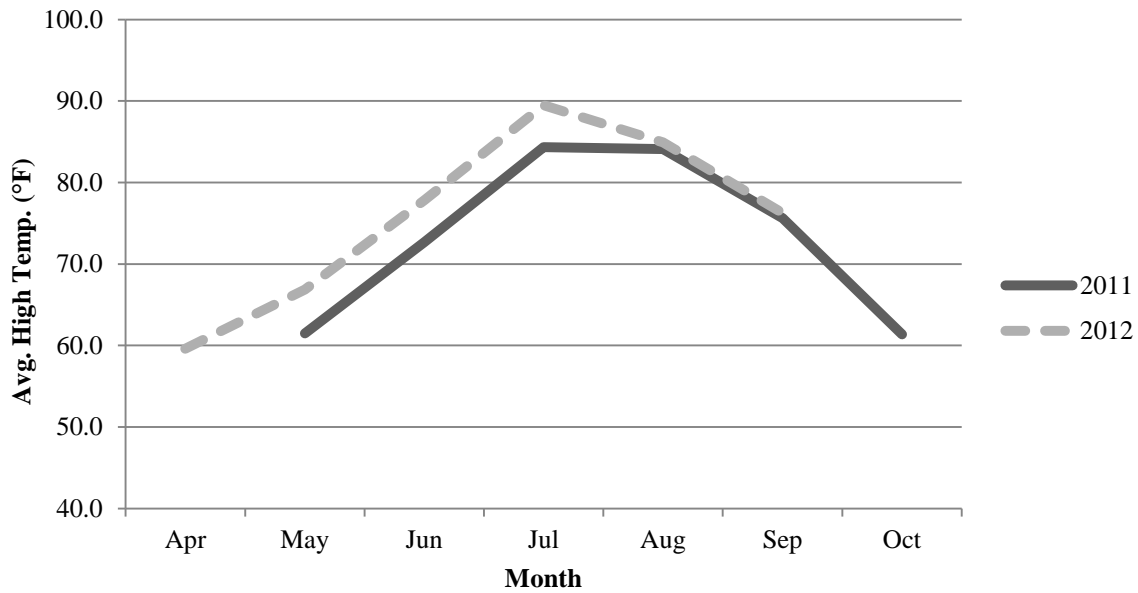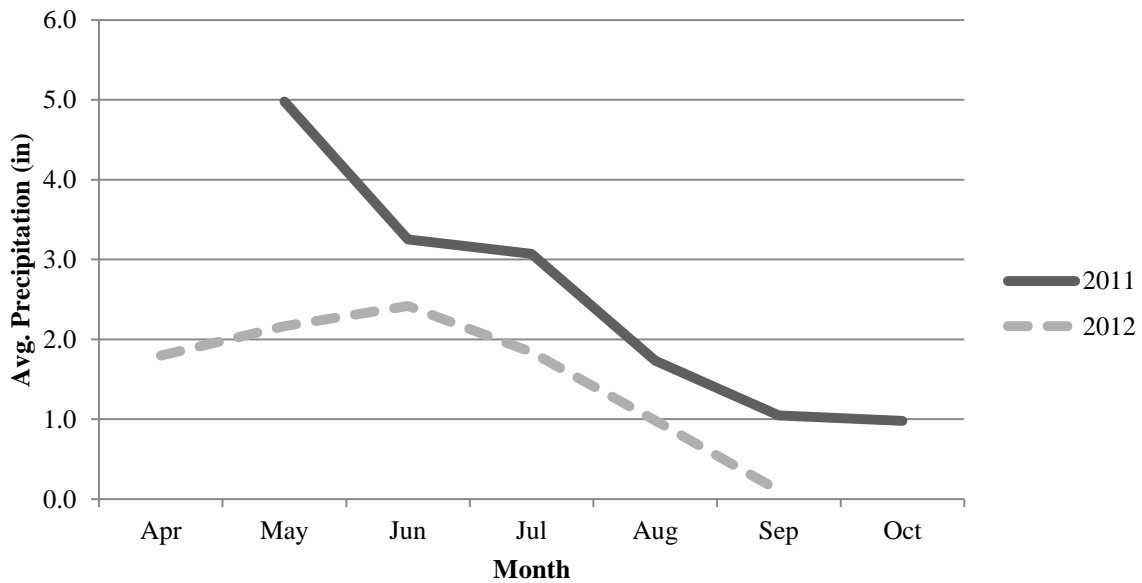
A.

B.

*Averages were calculated based on data from one weather station selected per crop reporting area in each state (*3, 4, 25*). This data set consisted of N=6 (Montana (n=2); North Dakota (n=4)).

Figure 2.2. Average* high temperature (A) and precipitation (B) per month during the hard red spring wheat planting, growing, and harvest seasons in 2011 and 2012.

A.

B.

*Averages were calculated based on data from one weather station selected per crop reporting area in each state (*5, 6, 25*). This data set consisted of N=15 (Minnesota (n=2); Montana (n=4); North Dakota (n=6); South Dakota (n=3)).

Figure 2.3. Average* high temperature (A) and precipitation (B) per month during the barley planting, growing, and harvest seasons in 2011 and 2012.
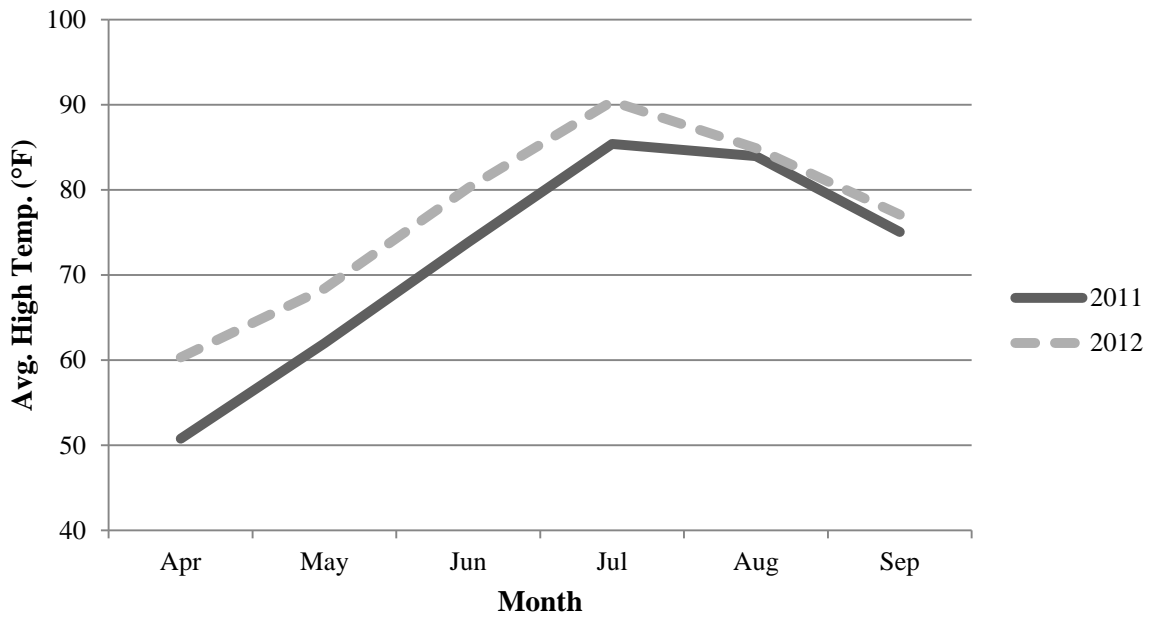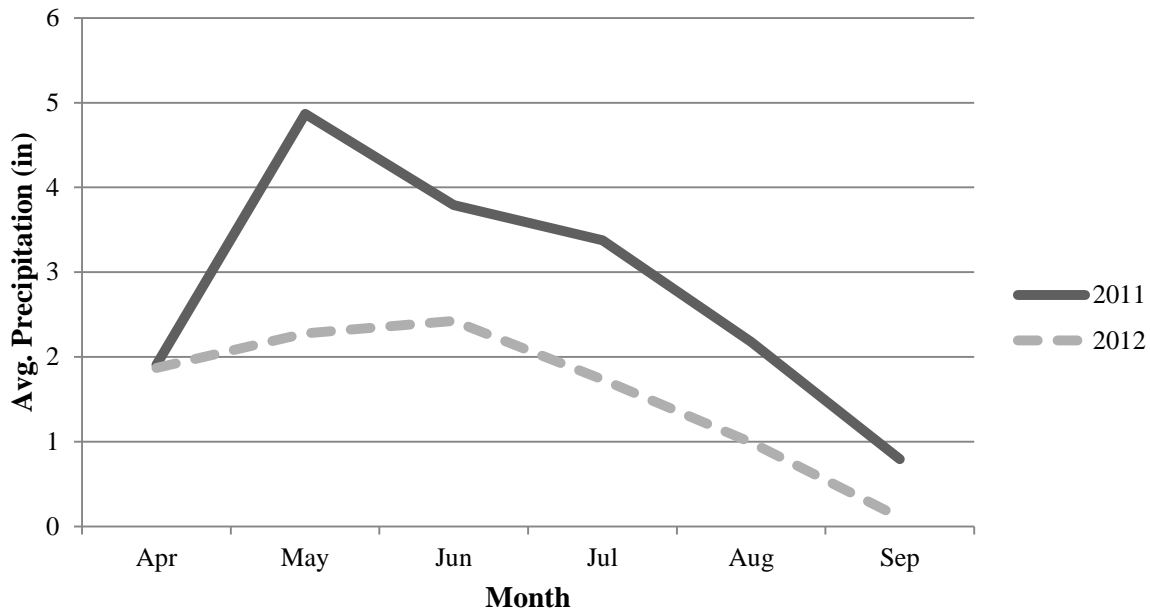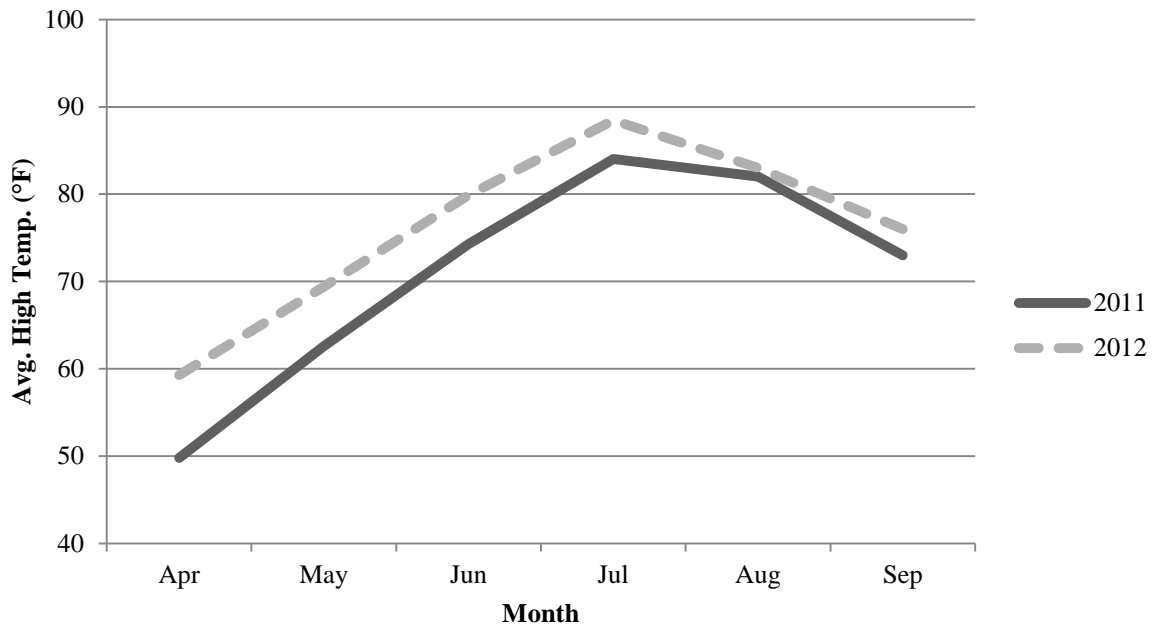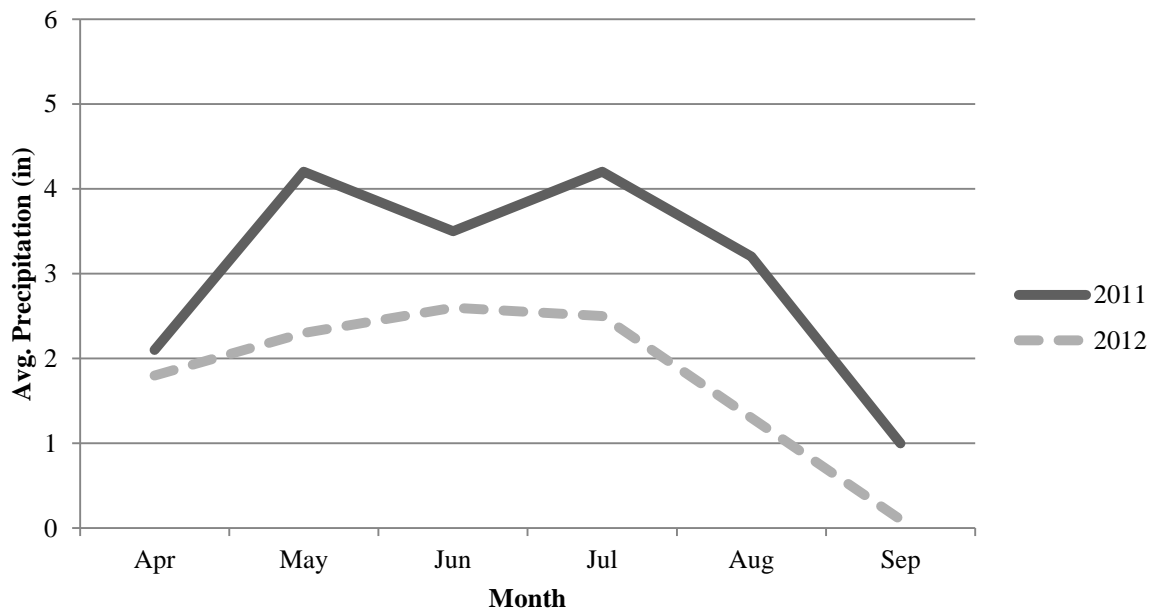
A.

B.

*Averages were calculated based on data from one weather station selected per crop reporting area in each state (*2, 25*). This data set consisted of N=10 (Minnesota (n=2); North Dakota (n=8).

Czerwiecki et al. (*10, 11*) analyzed Polish rye, barley, and wheat in 1997 and 1998. OTA was more prevalent and found at higher levels in 1998 than in the previous year for all grain types. It was proposed that precipitation may have been a contributing factor as the average rainfall in the area was 100 mm higher in 1998 than in 1997 (*11*). Given that the association between weather and OTA prevalence has been based on just 2 years of data in the aforementioned studies, it remains inconclusive as to whether the differences are indeed weather related or can be attributed to natural variation between years.

The Danish food-monitoring system has screened for OTA in Danish wheat and rye grain and flour over a period of 14 consecutive years (*18, 19*). A total of 475 wheat samples were tested between 1986 and 1992 of which 135 (28.4%) had detectable levels (>0.05 ng/g) of OTA. Nine samples had ≥5.0 ng/g OTA (*19*). Between 1992 and 1999, 419 wheat samples were tested and 191 (45.6%) were detected at >0.01-0.08 ng/g. Four samples had ≥5.0 ng/g OTA (*18*). The authors state that in 1986-1992 there was a clear relationship between OTA concentrations and weather conditions in that average to wet years resulted in higher OTA concentrations than in dry and very dry years (*19*). Between 1992-1999 the correlation was less clear as the harvest years between 1992 and 1997 were characterized as average to very dry. Only 1998 was considered to be a wet harvest year. When comparing the OTA levels in 1998 with the wet years between 1986 and 1992 it was found that OTA levels were lower in 1998 than in previous wet years. This difference was attributed to improved grain-drying practices that had been implemented after issues with wet grain and the occurrence of OTA became evident in the mid-1980s (*18*). Given that OTA production in grain is a dynamic system, studies with the intent to elucidate the exact nature of the relationship between weather parameters during the planting, growing, and harvest seasons and OTA incidence over time are needed.

To our knowledge this is the first study that examines the incidence of OTA in freshly harvested durum wheat in the United States. This study contributes to current knowledge of OTA prevalence in barley, durum and HRS wheat produced in the United States. Annual surveillance of OTA in these commodities is recommended in order to establish a database which would aid in understanding annual variation in OTA occurrence and the levels at which the toxin is present.

Acknowledgments

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CHAPTER 3. OCHRATOXIN A IN NATURALLY STORED, UNITED STATES BARLEY, DURUM AND HARD RED SPRING WHEAT

Abstract

Ochratoxin A (OTA) is a mycotoxin of significant health concern that is present in a variety of cereal grains and other foods around the world. OTA contamination is largely a storage issue which can be controlled through the implementation of proper storage practices. Barley, durum and hard red spring wheat samples that had been stored for various lengths of time were collected (N=262) over a period of two years by multiple commercial grain companies located in the northwestern and Northern Great Plains regions of the United States. Samples were analyzed for OTA concentration using high-performance liquid chromatography (HPLC) with fluorescence detection (FLD). OTA was detected in 12.2% of the samples and of those samples 81.3% had been stored for ≥6 months. One sample of barley and 4 of wheat exceeded 5 ng/g OTA.

Introduction

Ochratoxin A is a toxic secondary metabolite produced by fungi of the genera *Aspergillus* and *Penicillium*. In cold and temperate climates, such as Europe, Canada and the United States, *Penicillium* species are the most common OTA producers whereas in tropical climates *Aspergillus* species predominate (*8, 22*). Naturally occurring OTA was isolated for the first time from a commercial corn sample in 1969 (*33*). Subsequently, OTA has been detected worldwide in animal feed and a variety of commodities and foods including oats, wheat, rye, barley, corn, fruits, coffee, spices, fruit juice, wine, beer, pork, poultry, milk, and infant formula (*1, 6, 17, 19,*

29, 30, 32, 34, 35). Humans are exposed to OTA either directly by eating foods contaminated

with OTA or indirectly by consuming meat or milk from animals fed with OTA-tainted feed

(*11*). Toxicological studies have found OTA to be an immunosuppressant, nephrotoxic,

embryotoxic, teratogenic, neurotoxic, genotoxic, and it has been classified as a 2B carcinogen by

the IARC (*18*). According to the European Food Safety Authority and the Joint Committee

FAO/WHO of Experts on Food Additives the current maximum tolerable daily intake for

humans is estimated at 14 or 17.1 ng/kg of body weight, respectively (*12, 16*).

Cereal grains and cereal-based products, a diet staple in much of the world, account for

50-80% of total OTA intake by humans (*10, 14*). Ochratoxigenic fungi originate in the soil or on

decaying plant material and therefore are present in the field and on kernels prior to harvest,

during which time OTA may be produced (*25*). However, OTA contamination is more often a

result of grain harvested at high water content, improperly drying grain prior to storage, or

storage under humid conditions. As of 2012, at least 35 countries around the world regulate OTA

(*13*). In general, the maximum limit in unprocessed cereals is 5 ng/g OTA. The United States has

not set OTA guidance levels or limits for any food or commodity.

In the past, the focus of OTA surveys has been on commodities directly after harvest or

on commercially available foods. Another facet of research has examined the effect of various

storage conditions on ochratoxigenic fungi and OTA production (*3, 7, 24*). A few of these studies

tested the effects on grain stored in silos or other storage containers but most utilized bench-scale

models under controlled laboratory conditions. Even though it is clear that both of these research

avenues are significant and necessary, it is equally imperative that grain which has been stored

under natural conditions be surveyed in order to gain a holistic understanding of OTA occurrence

in the grain supply chain. The purpose of this study was to determine the prevalence and level of

OTA in a survey of barley, durum and HRS wheat grown in the major production regions of the United States and stored for various lengths of time under natural conditions.

Materials and Methods

Grain Samples

Barley (n=60), durum wheat (n=58), and HRS wheat (n=144) samples were collected on a voluntary basis at the point of receipt by several commercial grain companies. Participating companies were located in the following six states: Minnesota, Montana, North Dakota, South Dakota, Idaho, and Washington (Table 3.1.). Beginning in the fall of 2011 through the fall of 2012, participants were asked to provide one sample (~1 kg) per grain type (as applicable) per month. It was requested that the crop year and the date on which the grain was sampled at the company be provided for each sample. Storage length was determined by subtracting the first full month of harvest in the given crop year from the date the sample was received at the processor or elevator (Figure 3.1.). Each sample was mixed for homogeneity and 100 g sub-samples were milled for OTA determination. Upon receipt and prior to analysis, samples were stored at -18°C.

Table 3.1. Number of stored barley and wheat samples obtained per state.

State	Barley n (%)	Durum Wheat n (%)	HRS Wheat n (%)
Minnesota	5 (7.7)	0 (0)	5 (3.5)
Montana	3 (5.0)	0 (0)	13 (9.1)
North Dakota	51 (82.3)	58 (100)	102 (71.3)
South Dakota	0 (0)	0 (0)	18 (12.6)
Idaho	1 (1.5)	0 (0)	0 (0)
Washington	0 (0)	0 (0)	5 (3.5)
Total (N)	60 (100)	58 (100)	144 (100)

Figure 3.1. Number of stored barley and wheat samples obtained per storage time (months).
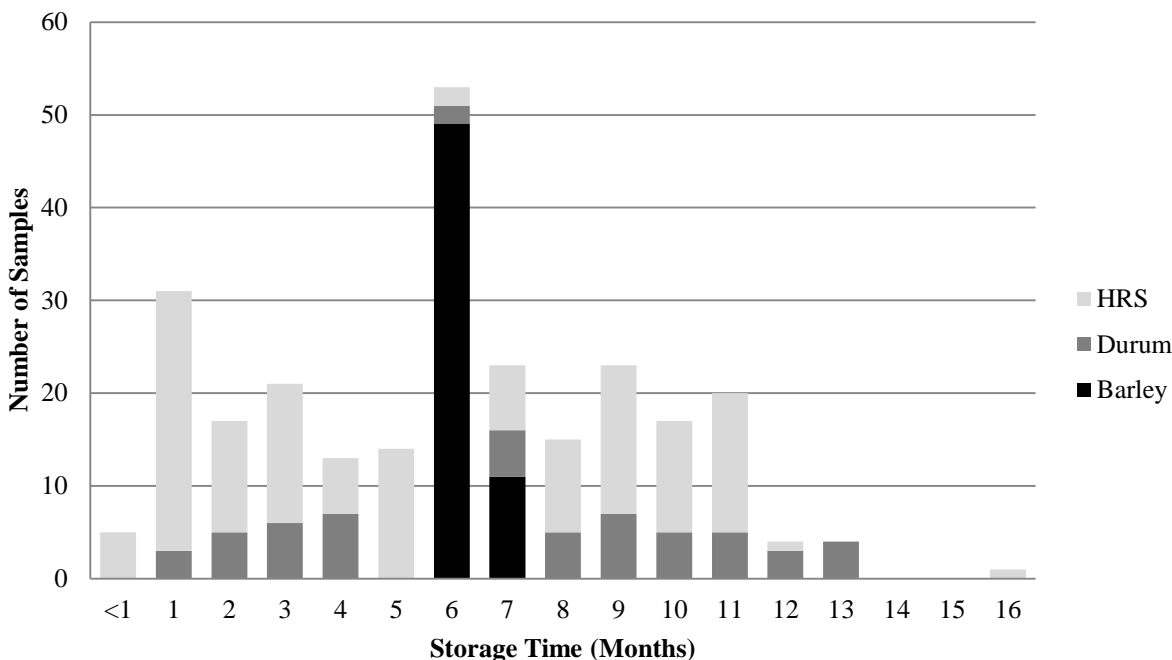
Ochratoxin A Determination

Samples were analyzed using AOAC method 991.44 (*27*) with a few minor alterations to the procedure (*20, 26*). Samples were milled to a fine powder (Perten Instruments, Model #3600, Hägersten, Sweden) for OTA determination. Sub-samples were mixed and stored at -18°C in reclosable 2mil plastic bags until analyzed. Samples were defrosted and allowed to warm to 25°C prior to analysis. A 50 g portion of the ground sample was added to a 500 ml media bottle, along with 25 mL 0.1M phosphoric acid, 250 mL chloroform, and then capped. The contents were shaken at ~175 RPM (25°C) for 80 min. Diatomaceous earth (~10 g) was added and shaken to mix. The extract was filtered and each sample filtrate was transferred to two centrifuge tubes, 5 mL of 3% sodium bicarbonate was added to each tube, and then shaken to mix. The emulsion was centrifuged at 851 x *g* for 5 min and the upper phase was collected and frozen until final extraction. Samples were allowed to thaw while columns were prepared. Five ml of sodium bicarbonate extract was cleaned with a Strata C18-E column (Phenomenex, Inc.,

Torrance, CA). The column was then washed and OTA eluted. The upper phase was transferred

to a secondary test tube and evaporated just to dryness under nitrogen. The residue was

dissolved in 500 µl mobile phase, capped and vortexed. The solution was filtered using a 0.45

µm microfilter (Pall Corporation, Port Washington, NY) into a 1.5-mL HPLC vial. Confirmation

of OTA was done by preparing its methyl ester.

A Shimadzu (Shimadzu Scientific Instruments, Columbia, MD) high-performance liquid

chromatography (HPLC) system (LC-20A) set-up consisted of a gradient pump (LC-20AD),

autosampler (SIL-20A HT) and fluorescence detector (RF-20A). The column (Phenomenex,

Inc., Torrance, CA) was 250 mm x 4.6 mm (i.d.) packed with 5 µm C18 bonded silica gel. The

analysis was performed under isocratic conditions using a mobile phase of acetonitrile-water-

acetic acid (51+47+2, v/v/v) at a flow rate of 1.0 mL/min. The fluorescence detector was set at

an excitation wavelength of 333 nm and an emission wavelength of 464 nm. An injection

volume of 25 µL was used and the retention time was approximately 10 min. The column oven

(CTO-20A) was set at 40.0°C.

A standard solution of OTA (Sigma-Aldrich, St. Louis, MO, USA) was prepared in

methanol according to the concentration established with a calibrated UV spectrophotometer.

The standard was evaporated to dryness and then dissolved in mobile phase. A set of five

standard solutions were prepared in the range of 0.2 to 2.1 ng and a standard curve was

established daily. The calibration curve proved linear. The limit of detection (LOD) and limit of

quantification (LOQ) for each grain were the concentrations of OTA that resulted in a signal-to-

noise ratio of 3 and 10, respectively (*21*). For barley and HRS wheat, the LOD was 0.06 ng/g and

the LOQ was 0.19. The LOD was 0.09 ng/g and the LOQ was 0.30 ng/g for durum wheat.

Recovery rates were determined by spiking known blank samples with multiple levels of OTA.

Three replicates were performed each day on three separate days for a total of nine samples per level per grain type. Spiked samples were left for one hour for the solvent to evaporate prior to extraction. Recovery rates were similar to that reported by Larsson and Möller (*20*) and Nesheim et al. (*26*). Confirmation of samples positive at levels >1.0 ng/g OTA was performed by methyl ester formation (*27*).

Statistical Analysis

The binomial proportions obtained for each grain type were tested for significance between 2011 and 2012 with the Chi-square test using SAS software (SAS Institute, Inc., Cary, NC, USA).

<div align="center">

Results and Discussion

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Ochratoxin A was detected in all grain types in both 2011 and 2012. Overall, a total of 32 (12.2%) samples had detectable levels of OTA. In 2011, OTA was most prevalent in durum samples (29.7%) as compared to HRS (15.4%) and barley (8.2%). However, the positive barley samples had the widest range and highest mean OTA concentration followed by the HRS samples. Positive durum samples had the smallest OTA range and mean (Table 3.2.). OTA was detected in samples from North Dakota, South Dakota, Montana, and Minnesota. Within these states positive samples were not concentrated in any specific region. Neither the barley sample from Idaho nor the five HRS samples from Washington were positive.

In 2012 the number of OTA positive samples decreased for durum and HRS but increased for barley. This difference in proportions between years was significant (p<0.05) only for HRS. In addition, the OTA range was smaller for all grain types in the second year of

sampling. Durum was the only grain type that had higher median and mean OTA concentrations in 2012 as compared to 2011 values.

Table 3.2. Number (%) of stored barley, durum and hard red spring (HRS) wheat samples positive for ochratoxin A (OTA) (ng/g) per year.

Year	Grain Type	# Positive/Total (%)	OTA (ng/g)		
			Range	Median	Mean
2011	Barley	4/49 (8.2)	0.16-185.24	1.87	47.28
	Durum	11/37 (29.7)	0.17-14.94	1.87	2.74
	HRS	10/65 (15.4)	0.32-49.27	0.75	7.57
2012	Barley	2/11 (18.2)	0.19-2.93	1.56	1.56
	Durum	2/21 (9.5)	0.43-12.41	6.42	6.42
	HRS	3/79 (3.8)	0.31-2.46	0.42	1.06

OTA levels in the six positive barley samples ranged from 0.16-185.24 ng/g. The level of OTA in the 13 positive durum wheat and 13 positive HRS wheat samples ranged from 0.17-14.94 ng/g and 0.31-49.27 ng/g, respectively (Table 3.3.). In the European Union raw cereal grains must not contain more than 5.0 ng/g OTA (*15*). One barley sample (185.24 ng/g) and four wheat samples (durum - 12.41 and 14.94 ng/g; HRS - 21.41 and 49.27 ng/g) exceeded this limit.

Overall, 26 (81.3%) of the OTA-positive samples had been stored for ≥6 months (Figure 3.2.). Positive HRS samples ranged from 1-11 months of storage. Likewise, positive durum samples had been stored from 4-12 months. Barley samples were positive at both 6 and 7 months of storage.

The median concentrations in barley samples were similar for both 6 and 7 months of storage (Table 3.4.). The most contaminated sample (185.24 ng/g) had been stored for 6 months. The highest median concentration in durum samples occurred at 9 months and the second highest at 4 months. This was in large part due to a highly contaminated sample at each of those time points (14.94 and 12.41 ng/g, respectively).

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Table 3.3. Range (ng/g) and mean (ng/g) of ochratoxin A (OTA) in positive stored barley, durum wheat, and hard red spring (HRS) wheat.

Grain Type	Number of Samples	OTA (ng/g)	
		Range	Mean
Barley	54	<0.06	<0.06
	2	0.06-0.20	0.18
	1	0.21-0.30	0.21
	0	0.31-0.40	--
	0	0.41-0.50	--
	0	0.51-0.60	--
	0	0.61-0.99	--
	3	1.0-10.0	63.90
Durum	45	<0.09	<0.09
	2	0.09-0.20	0.18
	2	0.21-0.30	0.23
	0	0.31-0.40	--
	1	0.41-0.50	0.43
	0	0.51-0.60	--
	1	0.61-0.99	0.77
	7	1.0-10.0	5.85
HRS	131	<0.06	<0.06
	0	0.06-0.20	--
	0	0.21-0.30	--
	3	0.31-0.40	0.32
	2	0.41-0.50	0.45
	1	0.51-0.60	0.53
	4	0.61-0.99	0.83
	3	1.0-10.0	24.38

The lowest median levels were from samples stored for 10 and 11 months. In the case of HRS, the median OTA concentration increased slightly as the length of storage increased. Contaminated HRS samples at >5 ng/g had been stored for 8 (49.27 ng/g) and 11 months (21.41 ng/g).

To our knowledge this is the first survey study conducted in the United States that specifically targets OTA in barley and wheat stored for different lengths of time under natural conditions. In a Spanish study (*24*) 105 samples of stored barley were collected over a two-year period from 21 different grain storage units. Results showed that 20% of the samples were

positive containing 0.05-1.6 ng/g (mean = 0.47 ng/g). The maximum level detected was 2.0 ng/g.

The method used had a LOD of 0.05 ng/g and LOQ of 0.17 ng/g. In that survey all of the

positive samples had been harvested in the spring and then subsequently stored during the

summer (*24*).

Figure 3.2. Number of OTA positive stored barley and wheat samples per storage time
  (months).
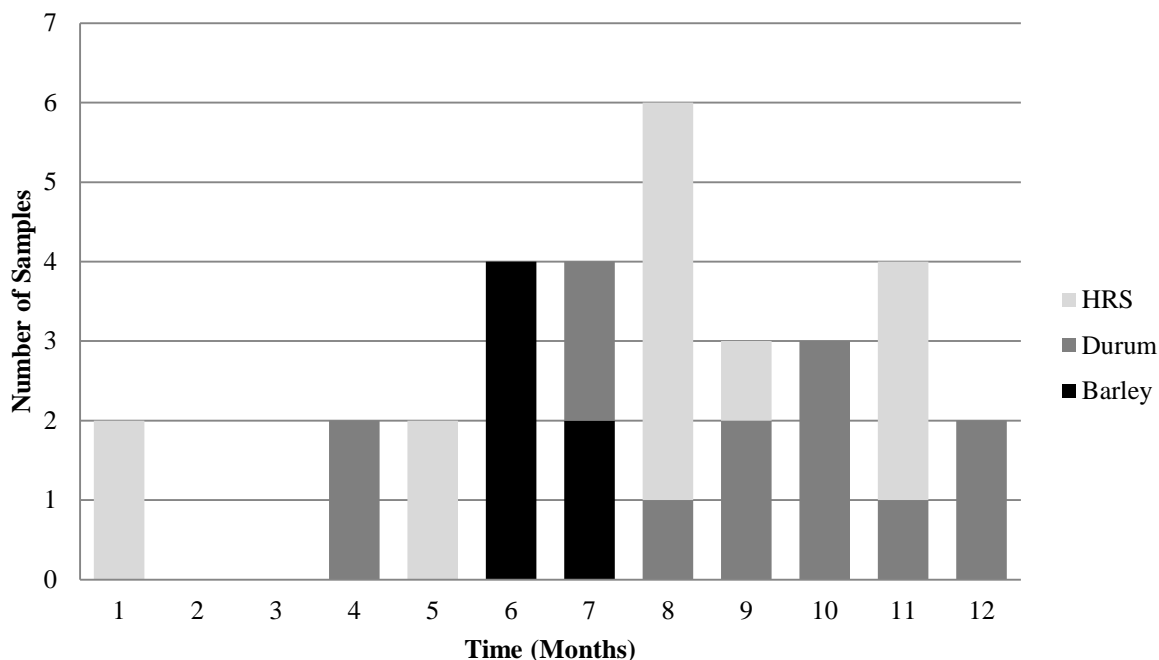
Table 3.4. Median OTA concentration (ng/g) per grain type over storage time (months).

Grain	Length of Storage (Months)											
Type	1	2	3	4	5	6	7	8	9	10	11	12
Barley	--	--	--	--	--	1.87[a]	1.56	--	--	--	--	--
Durum	--	--	--	6.42	--	--	2.39	1.87	8.42	0.23	0.18	1.39
HRS	0.42	--	--	--	0.45	--	--	0.73	0.79	--	0.92	--

[a] All barley samples obtained for analysis had been stored for either 6 or 7 months. Samples
  stored for any other length of time were not provided.

In early 2000 Prickett et al. (*31*) sampled British wheat (n=201), barley (n=106), and oats

(n=13) that had been harvested in 1999 and subsequently stored in silos, floor stores, and open

bins. Overall, OTA was detected in 16% of the samples. Barley had the highest incidence

(18.9%) while 15.9% of the wheat samples had detectable levels of OTA. OTA was not detected in any of the oats samples. Six wheat and five barley samples exceeded 5 ng/g OTA. The maximum OTA levels were 231.0 ng/g and 117.0 ng/g, respectively. The LOD was 0.1 ng/g.

Between October 1997 and June 1998, 306 stored wheat (n=148), barley (n=131) and oats (n=21) samples were collected from trucks and elevators in the United Kingdom and analyzed for OTA (LOD = 0.1 ng/g) (*32*). Oats had the highest incidence of OTA (28.6%) followed by barley (26.7%) and wheat (14.9%). The mean OTA concentration was 0.53, 2.60, and 1.94 ng/g, respectively. In general, the percentage of positive samples and the mean OTA concentration increased with storage time and moisture content. The results of the current survey confirm the general trend that the longer grain is stored the likelihood of OTA contamination increases. With that said, the length of storage should not be considered an absolute predictor of the level of contamination.

Given that 18.8% of OTA-positive samples in this study had been stored for only 1 to 5 months, it is evident that the storage conditions to which grain is subjected has a significant effect on the length of time grain can be stored safely. In storage studies, Abramson et al. (*4*) found that wheat and barley stored for 16 weeks at 15% initial moisture content (IMC) did not result in detectable levels of OTA. At 19% IMC OTA was detected in barley at 20 weeks (70 ng/g) but was not found in wheat. In a separate study durum wheat was stored at an IMC of 16% or 20% at 22°C in a granary for 20 weeks. OTA was detected at 4 weeks with maximum concentration being produced at 20 weeks. OTA was not detected in the 16% IMC samples (*3*). Birzele et al. (*7*) observed similar results after storing wheat at 20°C and 17% or 20% IMC. The samples that had been stored at 17% IMC resulted in no detectable OTA after 6 weeks. In contrast, the first set of 20% IMC samples OTA was detected after 2 and 4 weeks of storage.

However, in the following year, OTA was not detected in the 20% IMC samples after 6 weeks of storage.

In another study, Canadian barley, western red spring wheat, and western oats were stored at 21% IMC. By 4 weeks both the barley and wheat had detectable levels of OTA. After 20 weeks of storage OTA was still undetectable in oats. Final OTA concentrations were much greater in barley than in wheat (*5*). In a similar study, Abramson et al. (*2*) subjected hulless barley to storage at 15% and 19% IMC for 20 weeks. At 20 weeks the OTA concentration of 19% IMC barley was 24 ng/g and in 15% IMC barley it was not detected. Ideally, small grains should be dried to ≤14-14.5% moisture content immediately after harvest and provided sufficient air circulation and temperature and moisture control during storage in order to negate fungal growth and mycotoxin production (*23*).

The method of sampling utilized in this study is a limitation. An attempt was made to acquire samples that had been stored for a period between 1 and 12 months from multiple locations in each of the wheat- and barley-producing states in the northwest and Upper Great Plains regions of the United States. However, since participation was entirely voluntary, the distribution of where the samples ultimately were taken and the number of samples received per length of storage varied. The exact storage conditions for each sample used in this study were unknown. Storage length was the only information that was requested to be provided for these samples. Regardless, the information garnered in this study provides previously unavailable information about the prevalence and extent of OTA contamination of stored wheat and barley in the Upper Great Plains and northwestern regions of the United States.

It is recommended that future studies survey wheat and barley that is intended for use in food or animal feed, from all applicable regions in the United States, after various forms of

storage (e.g. silo, barge, elevator, etc.) prior to processing. Given that most forms of processing have only a minimal effect on reducing OTA, the resulting data would provide valuable insight as to the variation that occurs in the grain supply on a year-to-year basis, as well as the actual OTA levels entering the food chain and, ultimately, reaching the consumer.

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CHAPTER 4. INFESTATION AND QUANTIFICATION OF OCHRATOXIGENIC FUNGI IN BARLEY AND WHEAT NATURALLY CONTAMINATED WITH OCHRATOXIN A

Abstract

Multiple ochratoxigenic *Aspergillus* and *Penicillium* spp. have been reported as contaminants on various cereal grains around the world, although relatively few species dominate in any given location. Cereal grains are a significant source of ochratoxin A (OTA) in the human diet. Efforts to mitigate the risk of fungal contamination and OTA accumulation can be made pre- and post-harvest. Still, a rapid and reliable screening method is needed which can be used to predict the OTA level of a sample, and to inform risk assessments prior to processing. In this study, infestation rates were determined for freshly harvested and stored barley, durum and hard red spring wheat samples (N=139) with known OTA levels. Presumptive ochratoxigenic isolates were tested for their ability to produce OTA. The non-ribosomal peptide synthase (*otanps*PN) involved in OTA biosynthesis was used to quantify ochratoxigenic fungi in barley and wheat. Viable *Penicillium verrucosum* was present in 45% of the samples. In total, 62.7% (N=110) of the *P. verrucosum* isolates tested produced OTA on a microbiological screening medium. Both OTA level and infestation rate (*r*=0.30) as well as OTA level and *otanps*PN concentration (*r*=0.56) were weakly correlated. Neither infestation rate nor *otanps*PN concentration is a reliable predictor of OTA level in a sample. This work establishes that *P. verrucosum* is the primary OTA-producing fungi in wheat and barley from the northwestern and Upper Great Plains regions of the United States.

Introduction

Ochratoxin A is a toxic secondary metabolite produced by fungi of the genera *Aspergillus* and *Penicillium*. It is a relatively stable molecule that has carcinogenic, nephrotoxic, teratogenic, immunotoxic, and neurotoxic properties (*20*). Cereals and cereal-based products account for 50-80% of the daily intake of OTA in the human diet (*15*). Although it is widely viewed that the risk of adverse health effects due to OTA is low for the majority of the population, it is recognized that measures should be taken to reduce exposure. Currently over 99 countries around the world regulate OTA levels in an effort to protect consumer health and to ensure fair trade practices (*29, 52, 53*). Regulatory levels range from 0.5-50 ng/g, depending on the commodity or food product (*50, 53*).

Which OTA-producing fungi are present in a given commodity largely depends on the climate in which the crop is grown as well as the substrate. In cold and temperate climates such as Europe, Canada, and the northern United States, *Penicillium* species are the most common OTA-producing fungi. *Aspergillus* species occasionally are isolated from foods in temperate environments, but they are much more common in tropical climates (*36*). *P. verrucosum* is the only OTA-producing fungi to be reported to date in cereals and cereal products in northern Europe and Canada, and more recently in countries with warmer climates such as Italy, Spain, France, and Portugal. *A. ochraceus* also has been found, but it is considered a rare occurrence (*34*).

OTA contamination can occur during multiple stages of the grain supply chain: prior to harvest, during the harvesting process, drying and storage, and during some types of processing (*9, 36*). The level of fungal growth and subsequent OTA contamination that occurs on any given commodity depends on the environmental conditions (e.g. temperature, CO_2, water activity),

OTA-producing strain, endogenous microflora, as well as the commodity itself (*13, 29, 30, 46*). Pitt et al. (*42*) concluded that routine OTA analysis and the rejection of sample lots that do not meet standard specifications currently is the only effective means by which to reduce OTA in human food and animal feed at the point of processing. Therefore, it would be very useful if a correlation between OTA level and infestation rate or biomass could be established. This would allow for a relatively inexpensive but effective means by which grain could be screened anywhere along the food chain prior to processing.

Culturing is relatively inexpensive but is often labor intensive and time consuming. Dichloran yeast extract sucrose 18% glycerol (DYSG) agar is an accepted recovery and diagnostic medium for *P. verrucosum* in complex matrices such as soil and grain (*17*). It is a selective medium as it inhibits bacteria and fastidious fungi, but it also is differential because *P. verrucosum* is easy to distinguish from other *Penicillium* spp. based on its red-brown colony reverse (*34*). The reliability of DYSG in promoting secondary metabolite production such as OTA without the need for sub-culturing also has been established (*21*).

DNA-based assay techniques are an alternative to traditional culture techniques. Advantages include sensitivity, specificity, accuracy, and results that can be obtained within a day (*25*). However, sample preparation and DNA extraction can increase total analysis time to several days. The OTA polyketide synthase (*otapks*PN) and the non-ribosomal peptide synthetase (*otanps*PN) genes are considered the two primary genes encoding important enzymes in OTA biosynthesis (*1, 10, 27*). The *otanps*PN gene is required for OTA synthesis for *Penicillium* and *Aspergillus* species. Therefore this gene has been targeted in multiple studies as a means to quantify OTA-producing fungi irrespective of species (*10, 24, 43, 44*).

The objective of this study was to identify the primary ochratoxigenic species in naturally infested freshly harvested and stored barley, hard red spring wheat and durum wheat from the Upper Great Plains and northwestern regions of the United States. The relationship between infestation rates, OTA levels, and *otanps*PN concentration in OTA contaminated (>1 ng/g) and non-contaminated samples also was investigated.

Materials and Methods

Grain Samples

Freshly harvested barley, durum and hard red spring wheat samples (N=1370) were obtained in 2011 and 2012 by the offices of the Minnesota, Montana, North Dakota and South Dakota National Agricultural Statistics Service (*2, 3, 4, 5, 6*). All surveys were under the supervision of the Department of Plan Sciences at NDSU. Due to budget constraints in 2012, a regional barley sample survey was not conducted by the Department of Plant Sciences. Barley samples were obtained from commercial sources, but the locations sampled were similar to those used in the 2011 survey. Approximately 0.91-1.36 kg samples were collected throughout the harvest season directly from growers, farm bins and local elevators, and stored in sealed moisture-proof plastic bags. Sample collection was weighted based on the projected production number (bushels) for each county. Each sample was stirred to mix, and 100 g sub-samples were created. Approximately two-thirds of the kernels were milled for OTA determination and the remainder reserved.

Stored grain samples (N=262) were collected at the point of receipt by several commercial grain companies located in Minnesota, Montana, North Dakota, South Dakota, Idaho, and Washington. Each sample was stirred to mix and 100 g sub-samples were milled for

OTA determination and the remaining kernels reserved. Upon receipt and prior to analysis, samples were stored at -18°C. All grain samples were tested for OTA using high-performance liquid chromatography (HPLC) with fluorescence detection (FLD) following AOAC Official Method 991.44 (*39*). HPLC conditions are described below. The limit of detection (LOD) and limit of quantification (LOQ) for each grain sample were the concentrations of OTA that resulted in a signal-to-noise ratio of 3 and 10, respectively (*33*). The LOD was 0.06 ng/g and the LOQ was 0.19 ng/g for HRS wheat. For durum wheat, the LOD was 0.09 ng/g and the LOQ was 0.30 ng/g. Average recovery rates for barley, durum and hard red spring wheat spiked at 5, 20, 50 ng/g in triplicate on three separate days were 58.4 ± 13.3, 58.5 ± 14.8, and 46.3 ± 15.5, respectively.

Samples containing >1 ng/g OTA (n=19) were individually analyzed, whereas samples with undetectable levels of OTA were divided into a series of composite samples (n=120). Freshly harvested samples were composited based on sampling region within each state, whereas stored sample composites were composited by state. Each 40 g composite consisted of ≤22 samples representing pre-designated sampling regions within each state (Table 4.1). Individual samples were mixed thoroughly prior to sub-sampling and again after combination. All samples were stored at -18°C until analysis.

Fungal Strains

Penicillium verrucosum NRRL 965, *Aspergillus ochraceus* NRRL 5175, and *Fusarium graminearum* NRRL 28336 were obtained from the United States Department of Agriculture Agricultural Research Service (Peoria, IL) and maintained on malt extract agar (MEA) at 25°C as well as in glycerol at -20°C.

Table 4.1. Number of freshly harvested and stored composites per state and cereal grain.

Cereal Grain	State/# Regions	# Composites (Freshly Harvested/Stored)
Barley	Idaho/1	0/1
	Minnesota/1	1/2
	Montana/1	0/1
	North Dakota/5	6/4
Durum	Montana/2	5/2
	North Dakota/4	14/7
HRS	Minnesota/2	10/1
	Montana/5	14/2
	North Dakota/6	32/7
	South Dakota/3	7/2
	Washington/1	0/2
	Total	**89/31**

Culture Media

Dichloran yeast extract sucrose 18% glycerol (DYSG) agar contained 220 g glycerol (anhydrous), 20 g yeast extract, 150 g sucrose, 0.5 g $MgSO_4 \cdot 7H_2O$, 20 g agar, 0.002 g dichloran, 0.05 g chloramphenicol, 0.01 g $ZnSO_4 \cdot 7H_2O$, 0.005 g $CuSO_4 \cdot H_2O$, to 1 L vol. in distilled water. Final pH 5.6 ± 0.1.

Yeast extract sucrose (YES) agar (MP Biomedicals, Solon, OH) contained 5 g yeast extract, 30 g dextrose, 0.05 g each of: adenine, histidine, leucine, lysine, and uracil, 17 g agar, to 1 L vol. in distilled water.

Malt extract agar (MEA) (Hardy Diagnostics, Santa Maria, CA) contained 20 g malt extract, 20 g dextrose, 6 g peptone, 15 g agar, to 1 L vol. in distilled water. Final pH 5.5 ± 0.2.

All media were autoclaved at 121°C for 15 min.

Grain Infestation

Samples were plated without surface disinfection as described by Lund and Frisvad (*34*). A total of 100 kernels of each sample were directly plated onto DYSG agar plates (6

kernels/plate) and incubated upright in the dark for 7 days at 20°C. The number of infested

kernels with fungal growth exhibiting the characteristic red-brown reverse of *P. verrucosum* was

recorded. A representative number of presumptive *P. verrucosum* colonies were transferred onto

MEA agar and compared to a standard culture to verify the identity of the isolate as *P.*

verrucosum (*41, 45*).

OTA Production and Detection

A representative number of presumptive *P. verrucosum* colonies from the infestation

studies were tested for OTA production with modifications to the procedure described by

Bragulat et al. (*11*). A total of three agar plugs were removed from the central area of a colony

on DYSG, placed into a small vial and weighed. Methanol (600 µl) was added to each vial and

incubated for 60 min. at room temperature. The extract was filtered using a 0.45 µm micro-

syringe and analyzed by HPLC. If a colony tested negative the colony was transferred to DYSG

and incubated for 7 days and then retested for OTA production. Black-spored Aspergilli were

transferred to YES and incubated for 7-14 days prior to testing for OTA production. Total OTA

concentration in each sample was calculated using AOAC Official Method 991.44 (*39*).

The Shimadzu (Shimadzu Scientific Instruments, Columbia, MD) HPLC system (LC-

20A) set-up consisted of a gradient pump (LC-20AD), autosampler (SIL-20A HT) and

fluorescence detector (RF-20A). The column (Phenomenex, Inc., Torrance, CA) was 250 mm x

4.6 mm (i.d.) packed with 5 µm C18 bonded silica gel. The analysis was performed under

isocratic conditions using a mobile phase of 51% acetonitrile, 47% water, and 2% acetic acid

(v/v/v) at a flow rate of 1.0 ml/min. The fluorescence detector was set at an excitation

wavelength of 333 nm and an emission wavelength of 464 nm. An injection volume of 20 µl

was used and the retention time was approximately 10 min. The column oven (CTO-20A) was set at 40.0°C.

A standard solution of OTA (Sigma-Aldrich, St. Louis, MO, USA) was prepared in methanol according to the concentration established with a calibrated UV spectrophotometer. The standard was evaporated to dryness and then dissolved in methanol. A set of five standard solutions were prepared in the range of ~0.2 to 2.0 ng and a standard curve was established daily. The calibration curve proved linear ($R^2 \geq 0.99$). The limit of detection (LOD) and limit of quantification (LOQ) for the extract was the concentrations of OTA that resulted in a signal-to-noise ratio of 3 and 10, respectively (*33*). The LOD was 0.002 ng/g and the LOQ was 0.008 ng/g. OTA confirmation was performed by methyl ester formation (*39*).

Recovery levels were determined as described by Bragulat et al. (*11*) with the following modifications. A total of 5 ml DYSG was pipetted into a 55-mm Petri dish. Prior to solidification, the OTA standard was added at one of three levels (0.7, 1.4, 5.4 µg/ml). Three agar plugs were removed, placed into a tared vial and weighed. Extraction was performed using methanol and analyzed by HPLC as described above. All sample preparations were performed in triplicate and repeated thrice.

Sample Preparation and DNA Extraction

The procedure described by Demeke et al. (*16*) was used to prepare whole grain samples and to extract DNA, with a few modifications. Each sample (40 g) was ground in a coffee grinder for 90 s and then inverted 10X while shaking to mix further. The ground sample (0.20-0.22 g) was weighed into a 2-ml screw-cap tube. Four sub-samples were taken from each sample contaminated with >1 ng/g OTA whereas a single sample was taken from composite samples.

P. verrucosum NRRL 965 was 3-point inoculated onto MEA and incubated for 7 days at 25°C. Mycelial mats were harvested with sterile needles, transferred to a 2-ml tube until half full and stored at -80°C until extraction.

Two 0.25-inch ceramic spheres (MP Biomedicals, Solon, OH) were added to each tube. Samples were pulverized to a fine powder using a Retsch Mixer Mill MM 301 (Retsch GmbH, Haan, Germany) for 1 min at 30 Hz. Samples were centrifuged (Eppendorf, Hamburg, Germany, Model 5810) for 2 min at 18,506 x *g* to pull the sample down from the cap. Samples were stored at -20°C until extraction.

A volume of 1 ml sodium dodecyl sulphate (SDS) extraction buffer was added to each sample and then homogenized on the mixer mill for 30 s at 30 Hz. Samples were centrifuged at 15,294 x *g* for 5 min. The supernatant (750 µl) was transferred to a snap-top microcentrifuge tube and 215 µl 3 M potassium 5 M acetate solution (pH 4.6) was added. The tube was inverted to mix and then incubated on ice for 30 min. After incubation, samples were centrifuged at 15,294 x *g* for 15 min at 4°C. The supernatant (700 µl) was transferred to a new microcentrifuge tube, 500 µl cold isopropanol was added, and the tube was inverted 10X to precipitate the DNA. Samples were then centrifuged (Eppendorf, Hamburg, Germany, Model 5415R) for 1 min at 371 x *g*. The pellet was washed two times by filling the tube with cold 70% ethanol, centrifuging at 134 x *g* for 1 min, and decanting the supernatant. The third and final wash consisted of filling each tube with 70% ethanol, centrifuging for 1 min at 134 x *g*, and then decanting the supernatant. Tubes were inverted on a paper towel to remove excess ethanol.

Samples were dried using a Savant DNA120 SpeedVac® concentrator system (Thermo Scientific, Waltham, MA). Each sample was dissolved in 200 µl TE-RNase (20 µg/ml RNase A in TE, pH 7.4) and then incubated for 30 min in a 65°C water bath, manually mixing the sample

every 10 min. Samples were centrifuged at 16,168 x g for 10 min and the supernatant transferred to new microcentrifuge tube prior to storage at -20°C.

DNA concentration was quantified using the QuantiFluor® ONE dsDNA System (Promega Corporation, Madison, WI). Prior to qPCR analysis, 100 ng total genomic DNA was run on a 1.5% agarose gel to verify DNA presence in and quality of each sample.

PCR

Conventional polymerase chain reaction (PCR) amplification was used to detect the universal fungal β-tubulin gene in each of the samples and pure fungal DNA controls using TubF/R primers designed by Atoui et al. (*7*). Each reaction had a final volume of 25 µl, containing: 6 µl (25 ng/µl) template DNA, 0.05 µl of each primer (200 nM), 2.5 µl 10X PCR buffer, 2 µl MgCl₂ (25 mM), 2 µl dNTPs (2.5 mM), 0.5 µl Taq DNA polymerase (5 U/µl), and 9.9 µl sterile deionized water. PCR was performed in an Applied Biosystems® 2720 thermo cycler (Life Technologies, Grand Island, NY, USA). The amplification program consisted of 1 cycle at 94°C for 4 min, 35 cycles of 94°C for 45 s, 60°C for 45 s, 72°C for 45 s, and 1 cycle at 72°C for 10 min. Amplification products were analyzed by electrophoresis in 1.5% agarose gels using 0.5X TBE (Tris/Boric acid/EDTA) buffer at 40 V for 2 h. Gels were stained with ethidium bromide and visualized using UV transillumination. An amplicon of ~380 bp was obtained for *Penicillium* and *Aspergillus* strains.

Real-Time PCR

The primer pair F/R-npstr targeting the *otanps*PN gene, as reported by Rodríguez et al. (*44*), was used to amplify a 117 bp product. The 25 µl reaction mixture consisted of 12.5 µl 2X PerfeCta™ SYBR® Green SuperMix, ROX™ (Quanta BioSciences, Inc., Gaithersburg, MD, USA), 0.1 µl of each primer (400 nM), 4 µl template DNA (25 ng/µl), and 8.3 µl sterile

deionized water. Applied Biosystems 7900HT Fast Real-Time PCR System (Life Technologies, Grand Island, NY, USA) was used for amplification and detection using the following cycling protocol: 95°C 10 min; 40 cycles of 95°C for 15 s, and 60°C for 1 min followed by melting curve analysis from 60-95°C to verify the product.

Each run included a standard curve consisting of a dilution series of *P. verrucosum* NRRL 965 DNA (4 ng-0.4 pg) and a non-template DNA control. All standards and unknown reactions were performed in triplicate and repeated twice. The concentrations of unknown samples were calculated by Applied Biosystems® 7900HT System SDS software (Life Technologies, Grand Island, NY, USA) based on the standard curve.

Statistical Analysis

The binomial proportions obtained for infestation rates and OTA production were tested for significance with the Chi-square test. The two sample t-test with the Bonferroni correction and two-way analysis of variance (ANOVA) were used to compare means. The relationship between infestation rate and OTA level was analyzed by Pearson correlation. All analyses were run using SAS software (SAS Institute, Inc., Cary, NC, USA).

<div align="center">

Results and Discussion

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The number of samples infested with *P. verrucosum* was greater for samples containing >1 ng/g OTA (78.9%) as compared to OTA-negative composites (40%). This difference was significant ($p<0.005$). In both the OTA-positive and OTA-negative groups, samples that had been stored had a higher average infestation rate than freshly harvested samples (Table 4.2.). The average infestation rates for the OTA-positive group were not significantly different at a level of

P<0.05 between freshly harvested and stored samples. However, infestation rates were

significant (*p*<0.005) between freshly harvested and stored samples in the composited group.

Table 4.2. Total number of samples infested with presumptive *P. verrucosum* and average and median percentage (%) of kernels infested in freshly harvested (FH) and stored barley, durum and hard red spring wheat per sample group.

Sample Group (OTA Level)	# Samples Infested/Total (%)	Avg. (%) Infestation Rate (Median)		
		FH	Stored	Overall
Positive (>1 ng/g)	15/19 (78.9)	12.6 (2)	37.6 (28)	31.1
Composites (ND*)	48/120 (40.0)	0.6 (0)	4.0 (2)	1.4

*Not detectable

In the 19 OTA-positive samples infestation rates ranged from 0-94% (Figure 4.1.).

Samples containing >5 ng/g OTA (n=8) had infestation rates between 2-92%, with an average of

35.9% and median of 28.5%. The samples with <5 ng/g OTA (n=11) had an average infestation

rate of 27.5% and median of 28.0%. Of the samples with non-detectable levels of OTA (n=120),

48 (40%) were infested between 1-27%. Overall the majority of these samples were not infested

with *P. verrucosum*. The correlation between rate of infestation and OTA (ng/g) was 0.52. Upon

analysis it was determined that the sample containing 185.2 ng/g OTA was an outlier. When the

outlier was removed correlation decreased to 0.3.

The average recovery of OTA obtained at each of the spike levels was significant

between DYSG and YES (*p*<0.05) (Table 4.3.). Overall, recovery of OTA on DYSG was

significantly greater than on YES (*p*<0.0001).

A total of 62.7% (N=110) of the *P. verrucosum* isolates from OTA-positive and OTA-

negative cereal samples produced OTA on DYSG (Table 4.4. and Figure 4.2.). The observed

difference in the number of confirmed OTA producers between sample groups was significant

p<0.001.

Figure 4.1. OTA concentration (ng/g) in 19 OTA-positive samples and corresponding infestation rates (%).
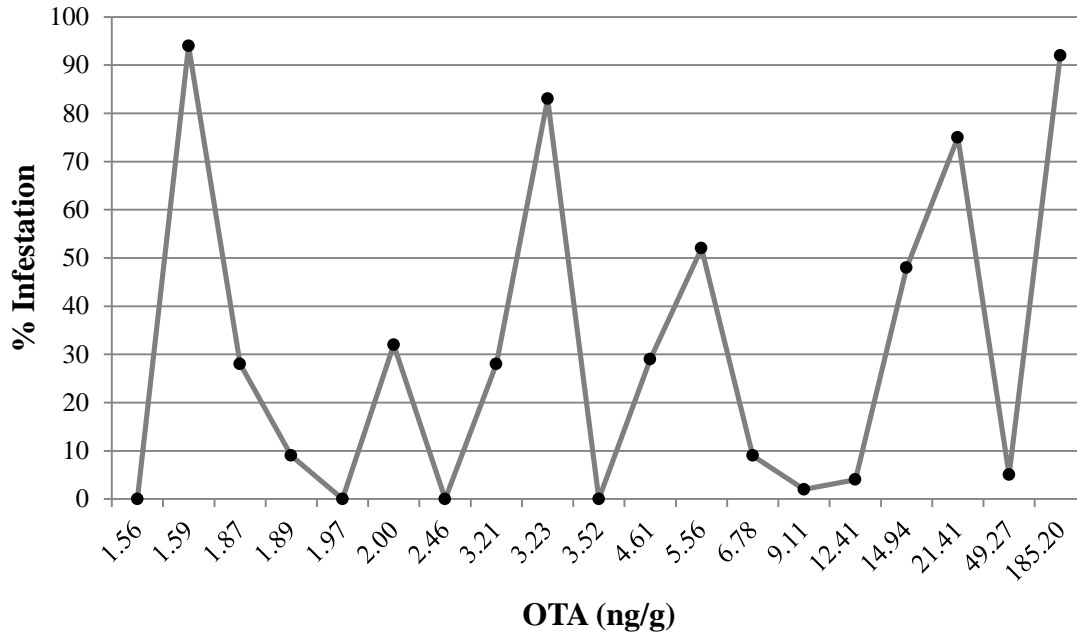
Table 4.3. Recovery of ochratoxin A (OTA)* in DYSG and YES media.

OTA Added (µg/ml)	YES Avg. Recovery, % (Range)	SD (%)	RSD (%)	DYSG Avg. Recovery, % (Range)	SD (%)	RSD (%)
0.7	27.5 (13.0-56.3)	14.4	52.4	46.1 (24.3-74.3)	15.1	32.8
1.4	59.3 (40.2-79.6)	11.4	19.3	77.5 (36.5-133.1)	34.2	44.2
5.4	76.2 (66.5-89.1)	8.2	10.8	100.8 (87.6-120.2)	11.5	11.5

*N=9 per OTA level

Table 4.4. Total number of presumptive *P. verrucosum* isolates tested and confirmed as ochratoxin A (OTA) producers.

Sample Group (OTA Level)	# *P. verrucosum* Isolates Tested	Confirmed (%)
Positive (>1 ng/g)	28	25 (89.3)
Composites (ND*)	82	44 (53.7)
Total	**110**	**69 (62.7)**

*Not detectable

Figure 4.2. Percentage of confirmed OTA producing and non-producing *P. verrucosum* isolates amongst samples containing >1 ng/g and non-detectable levels of OTA.
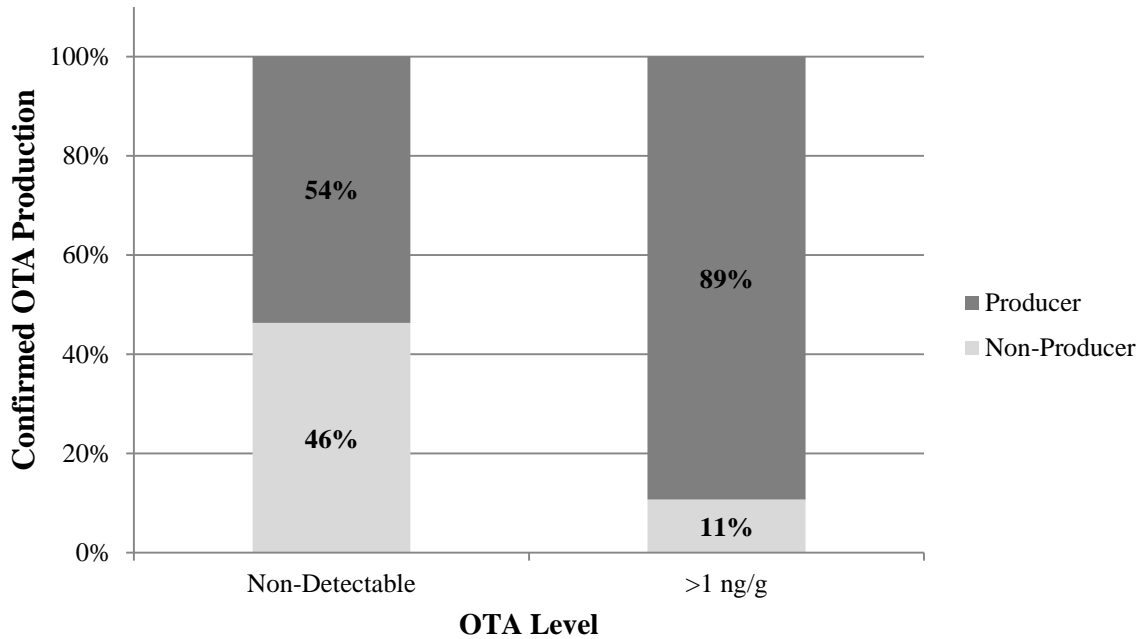
Two black-spored Aspergilli were isolated on DYSG and then transferred to YES agar for OTA determination. OTA concentration was calculated to be 13.0 ng/g after 14 d incubation (Figure 4.3.).

All samples were positive for the fungal β-tubulin gene. Nineteen cereal grain samples naturally contaminated with >1 ng/g were quantified for *otanps*PN. Four separate extractions were performed per sample. Each sub-sample was performed in triplicate twice. Each data point represents an average of 24 measurements. Eleven samples were below the detection limit (0.4 pg) of the assay. The coefficient of correlation was r=0.56 (Figure 4.4.).

Figure 4.3. HPLC-FLD chromatographs of *Aspergillus sp.* pure fungal extract after 14 d incubation on YES agar and OTA standards.
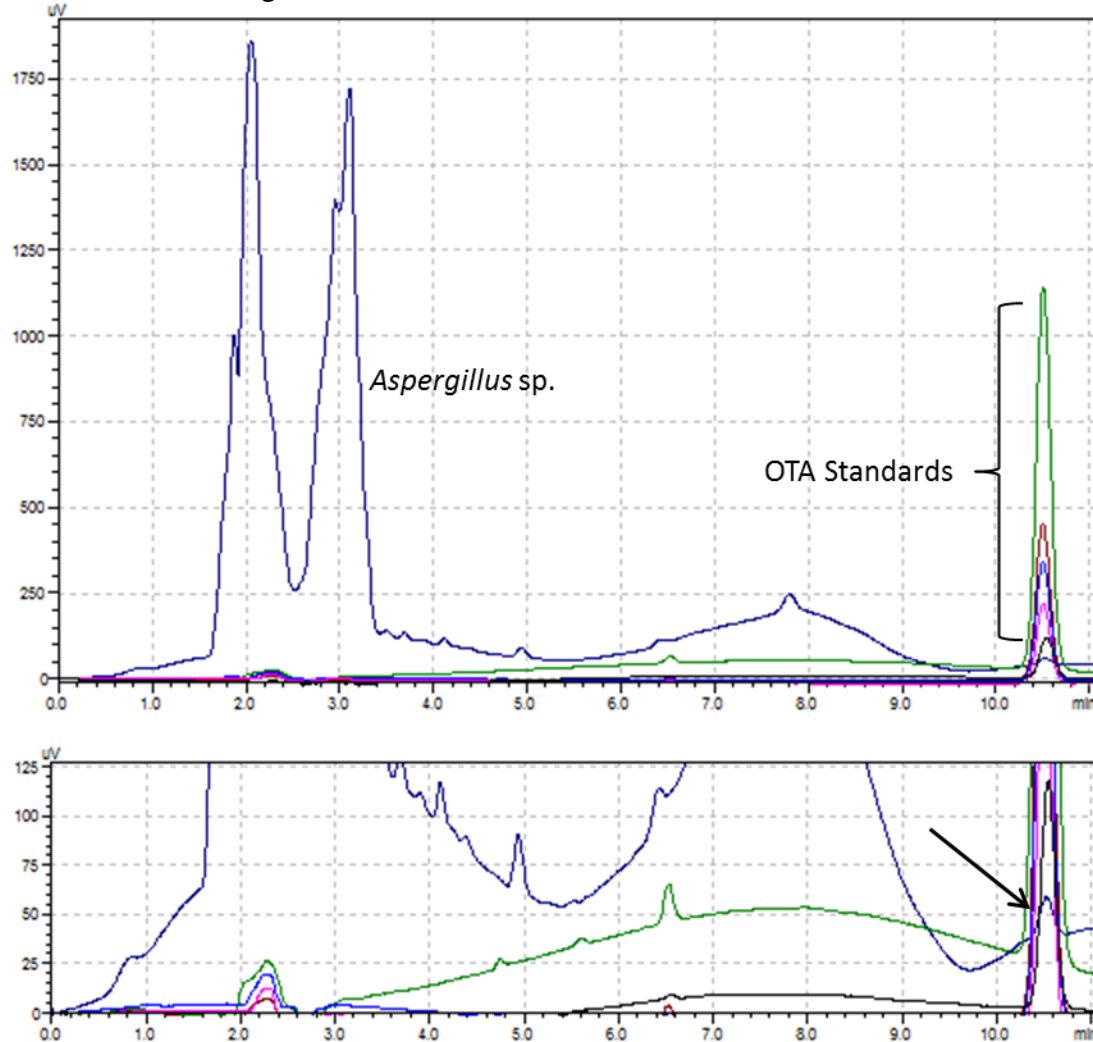
The majority (85%) of the 120 composite samples containing no detectable levels of OTA yielded results below the detection limit. Each sample consisted of six replicates. Eighteen samples had average detectable levels of *otanps*PN DNA (range=0.4-1.3 pg). Two of those samples contained extreme outliers averaging 200 and 532 ng *otanps*PN DNA.

Figure 4.4. OTA concentration (ng/g) and *otanps*PN DNA concentration (pg) of wheat and barley samples (n=8) containing >1 ng/g OTA.

$$y = 0.0021x + 0.6377$$
$$R^2 = 0.3137$$
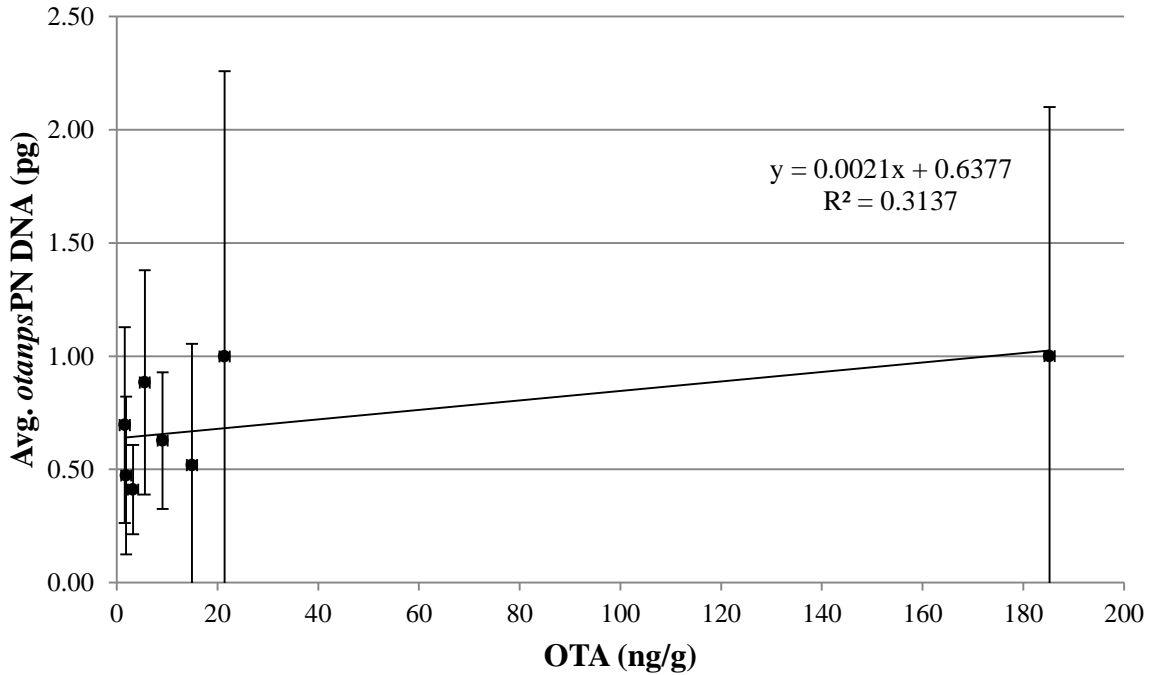
P. verrucosum is present on cereal grain in the field at relatively low levels (*38*). Elmholt (2003) analyzed combined grain prior to drying. *P. verrucosum* was isolated in 53% of the wheat, suggesting that the isolates could have originated from the soil during the harvest process or from farming equipment contaminated with conidia (*17*). In 1998, 51% (N=35) of combined samples were contaminated with *P. verrucosum* ranging from 0.6-5.8% infestation per sample (*18*). Another study found 82% (N=78) of combined grain samples prior to drying were contaminated with *P. verrucosum*, each at ≤58.7% infestation per sample. Three (3.8%) of the non-dried field samples had detectable OTA levels.

In 2003 Lund and Frisvad (*34*) analyzed *P. verrucosum* infestation rates in wheat and barley samples in order to determine if kernel infestation rate of *P. verrucosum* on DYSG can serve as an indicator of the presence of OTA. It was found that samples without OTA (n=11) had a maximum infestation rate of 4% whereas the 19 samples containing >5ng/g OTA all had >7% infestation. Although no direct correlation between these two factors was established, it was

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concluded that >7% *P. verrucosum* infestation would "strongly indicate" OTA production. In a subsequent study, Elmholt and Rasmussen (*19*) repeated Lund and Frisvad's methodology and found that 52% of their OTA-negative samples exceeded 7% infestation. No linear correlation between rate of infestation and OTA was established.

In this study, similar average infestation results were obtained for samples without detectable OTA levels. The average infestation rate was 1.4%, with a lower average (0.6%) in the freshly harvested samples than in the stored samples (4.0%). The maximum infestation rate for freshly harvested samples without detectable OTA was 5%. Overall, 11 (9.2%) of the OTA-negative samples were infested at 4-5%. Of eight samples contaminated at >5 ng/g OTA, three (37.5%) had <7% infestation (2, 4, 5%).

Given that 40% of all OTA negative composites were infested, clearly the lack of detectable OTA does not indicate absence of *P. verrucosum.* However, the total number of *P. verrucosum*-infested samples was significantly greater for OTA positive samples (78.9%) than OTA negative composites (40%). Still, these results indicate that no statistically significant correlation (*r*=0.30) exists between infestation rate and OTA concentration in these samples.

Infestation rate may not be a reliable predictor of OTA level in a sample, perhaps because OTA can long outlast the fungi that produced it, but the fact remains that early contamination of grain with *P. verrucosum* implies that the grain is at a greater risk of OTA contamination if subjected to improper storage conditions. Lund and Frisvad (*34*) pointed out that such a correlation may be hindered by the complexity of the grain microbiome and the microbial interactions that take place on that level.

It should be considered that the aforementioned conclusions have been based on relatively small sample sizes. Studies that investigate *P. verrucosum* infestation rates in a larger

number of cereal grain samples representing a broad range of OTA contamination analyzed both by individual cereal type as well as grouped as a single population would be essential to either confirm or refute conclusions using infestation rate as an indicator of OTA presence at any level. Also, given the challenges to obtaining homogenous samples, and that 'hot spots' are known to be an issue with cereal grain sampling, it may be worth exploring the variability in *P. verrucosum* infestation values based on plating only 100 kernels from a sample versus plating larger sample sizes.

A representative number of presumptive *P. verrucosum* isolates were selected from the infestation studies to undergo screening to confirm OTA production *in vitro*. Bragulat et al. (*11*) described a straightforward agar plug method, based on YES and Czapek yeast agar (CYA) media for screening OTA production in ochratoxigenic *Aspergillus* isolates. The optimal medium on which to test OTA production is heavily dependent on the target organism. As the samples in this study were grown in a temperate region, it was expected that *P. verrucosum* and not *Aspergillus* sp. would be the predominate OTA-producing organism. DYSG was chosen for infestation studies and for assessing OTA production as it is considered a diagnostic medium given the unique characteristics of *P. verrucosum* growth and that OTA is produced on this medium (*21*).

Since Bragulat et al. (*11*) did not disclose the number of replicates or any corresponding standard deviation values used in their recovery studies, a direct comparison between recovery values could not be conducted. Even though DYSG was the medium of interest for our purposes, recovery values for both DYSG and YES media were obtained for comparative purposes. It was found that recovery of OTA was significantly ($p<0.05$) greater on DYSG than YES. This work demonstrates that direct testing of agar plugs from presumptive *P. verrucosum* isolates on DYSG

is a reliable and easy means by which to confirm OTA production from this species. Although the focus of our work did not entail further optimization of the method, additional experimentation toward increasing extraction efficiency may be of value.

It has been demonstrated that the ability to produce OTA can be highly variable among isolates of the same species (*40*). This was supported in a study by Frisvad et al. (*22*) when only 16% of amplified fragment length polymorphism (AFLP) haplotypes of *P. verrucosum* isolates tested included >1 isolate. It is believed that the genes required for producing OTA are present in all *P. verrucosum*, but that in some cases it is silent, or that it requires specific environmental conditions for expression (*8, 22*). In the same study, it was reported that 74% (N=321) of *P. verrucosum* barley, wheat, and oats isolates produced OTA. Larsen et al. (*31*) found that 79% of *P. verrucosum* isolates tested (N=48) produced OTA. Seventeen *P. verrucosum* isolates were obtained from Spanish white wheat flour samples. OTA production was confirmed in 64.7% of the isolates (*12*).

Given these reports, it was anticipated that not all presumptive OTA-producing fungi in our study would produce OTA *in vitro*, and in fact our results confirmed this suspicion. A total of 62.7% (N=110) of presumptive *P. verrucosum* isolates from OTA-positive and OTA-negative cereal samples produced OTA on DYSG. Interestingly, the percentage of confirmed OTA-producing *P. verrucosum* isolates was significantly ($p<0.001$) greater for the OTA-positive samples than for the samples with undetectable levels of OTA.

This trend may partially explain why the OTA-positive samples contained higher levels of OTA; and, it also sheds light on why only weak correlations have been observed between infestation rate and OTA level. In other words, the concentration of *P. verrucosum* alone may not predict likelihood of contamination. Instead, more scrutiny should be placed on the percentage of

the infesting strains that are able to produce OTA and how that metric relates to level of contamination. The more a sample is infested with *P. verrucosum*, the greater the likelihood is that a higher percentage of isolates will produce OTA based on the concentration of *P. verrucosum* alone.

Another factor that may have affected OTA production on DYSG includes both the density and type of competing organisms. Although DYSG is a selective medium, *P. verrucosum* colony growth may have been limited as negative control samples had background levels of infestation averaging ~10^4-10^6 CFU/g (data not shown). This interaction has not been studied in detail with *P. verrucosum* in cereal grains; however, the effects of endogenous microflora on OTA production have been studied with *A. carbonarius* on grapes. It was found that in the presence of microbial competition, such as non-toxigenic *A. niger*, OTA production by *A. carbonarius* was inhibited at 30°C but not at 20°C, the organism's optimal temperature for OTA production. The result observed at 30°C was linked to multiple factors including growth restriction, consumption of specific nutrients required for OTA synthesis, degradation of produced OTA by flora, and excretion of compounds that block OTA synthesis (*51*).

Storage length also influences OTA production. During a 6-week storage study, Schmidt-Heydt et al. (*48*) observed that wheat inoculated with *P. verrucosum* exhibited low OTA production between 15-25 d, with the maximum concentration being reached after 25 d. In this case an increase in OTA production may have been a response to a decrease in available nutrients. In our study 14/19 (73.4%) of the cereal samples containing >1 ng/g OTA had been stored for some length of time.

The ability to predict the OTA level in a given sample based on *P. verrucosum* prevalence or colony counts would be advantageous, especially in terms of commercial

screening applications. Lindblad et al. (*32*) established that 10^2-10^3 CFU/g *P. verrucosum* on

DG18 agar served as an indicator that a sample likely was contaminated above the European

Union limit (5 ng/g OTA) in raw grains. Schmidt-Heydt et al. (*48*) confirmed the aforesaid

results in that all wheat samples containing >10^3 CFU/g *P. verrucosum* also contained OTA. The

one drawback of these methods is that they depended on viable *P. verrucosum* counts only. Since

OTA is stable over long periods of time it is not surprising that viable counts and OTA

concentration correlate poorly. This especially would be the case for stored cereal grain samples.

Since qPCR does not differentiate between viable and nonviable DNA, this issue becomes

partially resolved; but, it remains a potential source of variation.

Schmidt-Heydt et al. (*48*) developed qPCR primers targeting the OTA polyketide

synthase gene (*otapks*PV) for quantification of *P. verrusocum* in wheat. The purpose of the study

was to examine the growth kinetics of the organism over time using an initial inoculum level of

10^3 CFU/ml. The authors reported that the copy number of *otapks*PV "correlated well" with CFU

values obtained at each time point; however, a correlation coefficient was not provided.

Two papers have used F/R-npstr, the same primer pair that was used in this study, to

develop simplex and multiplex qPCR methods for the detection of ochratoxigenic fungi in foods

(*43, 44*). Reportedly this primer distinguished between OTA producing and non-producing fungi

regardless of genera. Rodríguez et al. (*44*) stated that the LOD of the qPCR method was 0.1 pg

based on a standard curve of pure *P. verrucosum* DNA and 1-10 conidia/g depending on the food

matrix (i.e. fruits, cooked meats, and ripened meats and cheese). The authors concluded that the

method could be used to quantify ochratoxigenic fungi in foods.

In the second paper, Rodríguez et al. (*43*) developed a multiplex TaqMan-based qPCR

test for quantifying aflatoxin-, patulin-, and OTA-producing fungi. Twelve commodities and

foods were artificially inoculated with spores of an aflatoxins, patulin, or OTA producer. Wheat was included in the study, but it only was used for quantifying *A. flavus*, an aflatoxin producer. LOD was provided for five of the matrices (not including wheat) and ranged from 10^1-10^3 CFU/g, depending on the specific combination of fungal strain and food matrix. The fungal load, as determined by a standard curve of foods inoculated with different levels of the mycotoxigenic mold, correlated (R^2>0.97) with plate counts on potato dextrose agar (PDA) (*43*). This work demonstrated that the multiplex qPCR method is an accurate means by which to quantify and monitor toxigenic fungi in foods. As both studies focused on method development, neither study explored using F/R-npstr to correlate the quantity of ochratoxigenic fungi in a sample with OTA concentration.

Schmidt et al. (*47*) did examine the relationship between *A. ochraceus* DNA content and OTA concentration in 30 samples of green coffee. OTA levels in the samples ranged from 0-72 ng/g. The LOD was 4.7 pg DNA/reaction. A correlation coefficient of 0.55 was obtained, and a positive correlation (*p*=0.01) between DNA concentration and OTA level was established. Three data points were outliers. One drawback of the method was that the chosen target sequence did not discriminate between OTA producing and non-producing strains of *A. ochraceus*.

The detection limit of our qPCR assay was calculated using serial dilutions of *P. verrucosum* NRRL 965 DNA and determined to be 0.4 pg genomic DNA/reaction. It is unknown at this time how many copies are present in the *Penicillium* genome. Genomic DNA concentrations <0.4 pg gave Ct value of 32.2±2.1. The real-time data showed that the Ct values correlated well (R^2 = 0.98-0.99) with DNA quantities ranging from 4 ng-0.4 pg. Eleven of the 19 samples (57.9%) having >1 ng/g OTA yielded results below the detection limit of the assay.

Based on this data, OTA concentrations are not correlated with *otanps*PN concentration ($R^2 =$ 0.31).

Obtaining a representative sample is crucial to the overall accuracy of a method. Sampling raw whole grains is especially challenging due to the heterogeneous nature of the product and mycotoxin production (*50*). This issue is exacerbated at the molecular level. This was evident in the form of %RSD values ranging from 48-126 for each of the samples that contained >1 ng/g OTA and were above the qPCR LOD. This variance may be reduced by increasing the number of samples or the sample size; however, upon implementation of the most robust plan a level of uncertainty still remains. Furthermore, even if adequate measures are taken to ensure the representativeness of each sample, results are ultimately dependent on the quality of the primer design.

During development of their primer set Rodríguez et al. (*44*) did not present data on screening against *Fusarium* species, a common fungus on cereal grains. In our studies, we found that *F. graminearum* NRRL 28336 cross-reacted with the primers, giving a melting curve that was indiscernible from OTA producers. After separating the PCR product using gel electrophoresis, a single band of 117 bp was produced, confirming the positive result. This is especially problematic, given the ubiquity of *Fusarium* ssp. on cereal grains in the North American grain producing region. This concern was allayed when it was determined that 0.9-9.0 ng of *Fusarium* DNA was needed to give a false-positive result (Ct value >34), a concentration that was highly unlikely to occur on non-visibly contaminated grain included in this study.

Our results highlight the need for primer sets that are specific for ochratoxigenic *P. verrucosum* in cereal grain. Quantitative PCR primers must be designed with the fungal target in mind as well as the food matrix to which the primers will be applied. Prior to use candidate

primer sets should undergo a comprehensive screening of isolates representing genera most likely to occur on wheat and barley. Undoubtedly, primers with better specificity will be developed as the OTA biosynthetic pathway becomes better understood.

It was expected that *P. verrucosum* would be the only OTA producer in these samples based on previous studies published using grain from a similar climate (*14, 23, 34*). All confirmed OTA-producers met the classic description of *P. verrucosum* on DYSG. In addition, two black-spored Aspergilli were isolated during infestation analysis. After incubation on YES for 14 d, one isolate was found to produce OTA. The level of OTA produced by the *Aspergillus* isolate was 2-3 orders of magnitude less than what was observed for pure *P. verrucosum* isolates on DYSG. Pure fungal DNA was extracted from the isolate and screened using PCR primers designed by Luque et al. (*35*) and the qPCR primers for *otanps*PN. The isolate was positive for both tests with a product at ~459 bp and Ct value of 18.0±0.3, respectively. Although other studies have reported isolating ochratoxigenic *Aspergillus* from grain, in all cases the isolates had not been properly confirmed or did not actually produce OTA (*34*). Both of the unknown *Aspergillus* isolates in this study were later identified at a collaborating laboratory using microscopic examination and PCR as *A. tubingensis,* a member of *Aspergillus* section *Nigri*.

A. tubingensis has had conflicting reports regarding its ability to produce OTA, but it is not currently believed to be an OTA producer (*37, 49*). It is possible that the isolated *A. tubingensis* strain has acquired the ability to produce OTA nominally. It also is possible that *A. tubingensis* may be producing a different metabolite which has a similar HPLC retention time to OTA. It is suspected that the PCR and qPCR results indicate that the *otanps*PN primers are not entirely representative of a complete OTA biosynthetic pathway. Testing the same isolate against a panel of primer sets that code for different parts of OTA biosynthesis would be valuable.

Another determinant step would be to analyze the fungal extract using instrumentation with higher resolution capacity, such as LC-MS/MS.

This work establishes that *P. verrucosum* is the primary OTA producer in wheat and barley in the Upper Great Plains and northwest regions of the United States. A DYSG-based agar plug method using methanol extraction is a reliable means to determine *P. verrucosum* OTA production *in vitro*. Although neither infestation level nor *otanps*PN concentration correlated with OTA level, studies that examine the relationship between the concentration of ochratoxigenic *P. verrucosum* and OTA level are warranted.

Acknowledgements

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CONCLUSION

These results show that OTA is produced on wheat and barley grown in the northwestern and Northern Great Plains regions of the United States prior to harvest at rates similar to what has been reported in Europe and Canada. However, direct comparisons are hindered by sample size, sampling methodology, and OTA extraction and detection methodology. This study supports the established fact that risk of OTA contamination increases over time. Testing samples for infestation rate or *otanps*PN concentration would be a quick and convenient way to assess OTA level; however, a strong correlation was not established. Instead, the ratio of OTA-producing to non-producing strains in a given sample may better indicate OTA level. *Penicillium verrucosum* is the primary OTA-producing fungi on barley and wheat in the Upper Great Plains and northwestern regions of the United States.

RECOMMENDATIONS

Annual surveillance of OTA in barley and wheat produced in the United States is recommended. Maintaining the data gathered from survey work in a database would provide insight into the natural variation in OTA prevalence that occurs annually. Multi-year surveillance and testing would allow us to gain a more holistic understanding of the levels at which OTA naturally occurs in United States barley and wheat.

Given that OTA production in grain is a dynamic system, studies with the intent to elucidate the exact nature of the relationship between weather parameters during the planting, growing, and harvest seasons and OTA incidence over time are needed. Trends that emerge from surveillance databases may help pinpoint key environmental parameters or interacting factors that result in increased OTA contamination.

Future studies should test wheat and barley that is intended for use in both food and animal feed, from all applicable regions in the United States, after various forms of storage (e.g. silo, barge, elevator, etc.) and prior to processing. Given that most forms of processing have only a minimal effect on reducing OTA, the resulting data would show the variation that occurs in the grain supply on a year-to-year basis, as well as the actual OTA levels entering the food chain and, ultimately, reaching the consumer.

Obtaining truly homogenous cereal grain samples remains a challenge. Studies that investigate *P. verrucosum* infestation rates in a larger number of cereal grain samples contaminated at a broad range of OTA levels, both by individual cereal type as well as grouped as a single population are warranted. The data would strengthen statistical significance of the relationship between infestation rate and OTA presence. Along the same lines, the variability in

P. verrucosum infestation values based on the traditional method of plating only 100 kernels from a sample versus plating larger sample sizes should be investigated.

Finally, our results highlight the need for primer sets that are specific for ochratoxigenic *P. verrucosum* in cereal grains. Quantitative PCR primers must be designed with the fungal target in mind as well as the food matrix to which the primers will be applied. Prior to use, candidate primer sets should undergo a comprehensive screening of isolates representing genera most likely to occur on wheat and barley. Undoubtedly, primers with better specificity will be developed as the OTA biosynthetic pathway becomes better understood.

APPENDIX

Table A.1. HPLC-FD results for 2011 barley survey samples.

Original Sample #	County	State	Julie Sample #	Analysis Date	Ret. Time[a] (min)	Area	Ht.[b]	OTA (ng/g)	Conf.[c]
				HPLC-FD					
1	Burke	ND	1	7/5/2012	ND[d]	--	--	--	--
2	Burke	ND	2	7/3/2012	ND	--	--	--	--
1	Divide	ND	3	7/5/2012	ND	--	--	--	--
1	Mountrail	ND	4	7/3/2012	ND	--	--	--	--
2	Mountrail	ND	5	8/20/12	ND	--	--	--	--
1	Renville	ND	6	7/5/2012	ND	--	--	--	--
2	Renville	ND	7	8/21/12	ND	--	--	--	--
1	Ward	ND	8	8/20/12	ND	--	--	--	--
2	Ward	ND	9	8/20/12	ND	--	--	--	--
3	Ward	ND	10	8/20/12	ND	--	--	--	--
4	Ward	ND	11	8/8/12	ND	--	--	--	--
1	Williams	ND	12	7/5/2012	ND	--	--	--	--
2	Williams	ND	13	7/5/2012	ND	--	--	--	--
3	Williams	ND	14	7/3/2012	ND	--	--	--	--
4	Williams	ND	15	8/20/12	ND	--	--	--	--
1	Benson	ND	16	8/20/12	ND	--	--	--	--
2	Benson	ND	17	7/5/2012	ND	--	--	--	--
3	Benson	ND	18	7/5/2012	ND	--	--	--	--
4	Benson	ND	19	7/5/2012	ND	--	--	--	--
5	Benson	ND	20	8/21/12	ND	--	--	--	--
6	Benson	ND	21	7/5/2012	ND	--	--	--	--
1	Bottineau	ND	22	7/6/2012	ND	--	--	--	--
2	Bottineau	ND	23	7/3/2012	ND	--	--	--	--
3	Bottineau	ND	24	8/21/12	ND	--	--	--	--
4	Bottineau	ND	25	7/5/2012	ND	--	--	--	--
1	McHenry	ND	26	4/27/2012	ND	--	--	--	--
2	McHenry	ND	27	4/26/2012	ND	--	--	--	--
3	McHenry	ND	28	4/27/2012	ND	--	--	--	--
4	McHenry	ND	29	4/27/2012	ND	--	--	--	--
1	Pierce	ND	30	7/5/2012	ND	--	--	--	--
2	Pierce	ND	31	8/8/12	ND	--	--	--	--
3	Pierce	ND	32	7/3/2012	ND	--	--	--	--
4	Pierce	ND	33	7/5/2012	ND	--	--	--	--
1	Rolette	ND	34	7/5/2012	ND	--	--	--	--
2	Rolette	ND	35	7/5/2012	ND	--	--	--	--

3	Rolette	ND	36	7/3/2012	ND	--	--	--	--
4	Rolette	ND	37	7/3/2012	ND	--	--	--	--
1	Cavalier	ND	38	4/27/2012	ND	--	--	--	--
2	Cavalier	ND	39	7/5/2012	ND	--	--	--	--
3	Cavalier	ND	40	7/6/2012	ND	--	--	--	--
1	Grand Forks	ND	41	7/6/2012	ND	--	--	--	--
2	Grand Forks	ND	42	8/24/12	ND	--	--	--	--
1	Nelson	ND	43	7/5/2012	ND	--	--	--	--
2	Nelson	ND	44	7/3/2012	ND	--	--	--	--
1	Pembina	ND	45	7/5/2012	ND	--	--	--	--
2	Pembina	ND	46	4/27/2012	ND	--	--	--	--
1	Ramsey	ND	47	7/5/2012	ND	--	--	--	--
2	Ramsey	ND	48	7/5/2012	ND	--	--	--	--
3	Ramsey	ND	49	7/5/2012	ND	--	--	--	--
4	Ramsey	ND	50	7/3/2012	ND	--	--	--	--
5	Ramsey	ND	51	4/27/2012	ND	--	--	--	--
1	Towner	ND	52	7/3/2012	ND	--	--	--	--
2	Towner	ND	53	8/20/12	ND	--	--	--	--
3	Towner	ND	54	8/20/12	ND	--	--	--	--
1	Walsh	ND	55	7/6/2012	ND	--	--	--	--
2	Walsh	ND	56	7/5/2012	ND	--	--	--	--
1	Dunn	ND	57	7/3/2012	ND	--	--	--	--
2	Dunn	ND	58	7/5/2012	ND	--	--	--	--
1	McKenzie	ND	59	7/3/2012	ND	--	--	--	--
2	McKenzie	ND	60	4/26/2012	ND	--	--	--	--
1	McLean	ND	61	7/10/2012	ND	--	--	--	--
2	McLean	ND	62	4/26/2012	ND	--	--	--	--
3	McLean	ND	63	8/8/12	ND	--	--	--	--
1	Mercer	ND	64	4/26/2012	ND	--	--	--	--
2	Mercer	ND	65	4/26/2012	ND	--	--	--	--
1	Oliver	ND	66	4/26/2012	ND	--	--	--	--
2	Oliver	ND	67	8/20/12	ND	--	--	--	--
1	Eddy	ND	68	7/9/2012	ND	--	--	--	--
2	Eddy	ND	69	8/8/12	ND	--	--	--	--
1	Foster	ND	70	4/27/2012	ND	--	--	--	--
2	Foster	ND	71	4/26/2012	ND	--	--	--	--
3	Foster	ND	72	8/21/12	ND	--	--	--	--
1	Kidder	ND	73	8/21/12	ND	--	--	--	--
2	Kidder	ND	74	4/26/2012	ND	--	--	--	--
1	Sheridan	ND	75	4/27/2012	ND	--	--	--	--

Table A.1. HPLC-FD results for 2011 barley survey samples (continued).

2	Sheridan	ND	76	4/26/2012	ND	--	--	--	--
3	Sheridan	ND	77	4/26/2012	ND	--	--	--	--
1	Stutsman	ND	78	7/3/2012	ND	--	--	--	--
2	Stutsman	ND	79	4/26/2012	ND	--	--	--	--
1	Wells	ND	80	7/10/2012	ND	--	--	--	--
2	Wells	ND	81	6/14/2012	ND	--	--	--	--
3	Wells	ND	82	7/6/2012	ND	--	--	--	--
4	Wells	ND	83	4/27/2012	ND	--	--	--	--
1	Clay	MN	84	8/24/12	ND	--	--	--	--
1	Kittson	MN	85	4/26/2012	ND	--	--	--	--
1	Marshall	MN	86	4/26/2012	ND	--	--	--	--
2	Marshall	MN	87	4/26/2012	ND	--	--	--	--
3	Marshall	MN	88	4/26/2012	ND	--	--	--	--
1	Pennington	MN	89	7/9/2012	ND	--	--	--	--
1	Polk	MN	90	4/27/2012	ND	--	--	--	--
2	Polk	MN	91	4/26/2012	ND	--	--	--	--
1	Roseau	MN	92	6/14/2012	ND	--	--	--	--
2	Roseau	MN	93	4/27/2012	ND	--	--	--	--

[a] Retention time of peak
[b] Height of peak
[c] Result of confirmatory test
[d] Not detected

Table A.2. HPLC-FD results for 2012 barley survey samples.

Original Sample #	County	State	Julie Sample #	HPLC-FD					
				Analysis Date	Ret. Time[a] (min)	Area	Ht.[b]	OTA (ng/g)	Conf.[c]
56	Spink	SD	B1	3/20/2013	ND[d]	--	--	--	--
57	LaMoure	ND	B2	3/20/2013	ND	--	--	--	--
58	Emmons	ND	B3	3/20/2013	ND	--	--	--	--
59	Stutsman	ND	B4	3/20/2013	ND	--	--	--	--
60	McPherson	SD	B5	3/20/2013	ND	--	--	--	--
61	Campbell	SD	B6	3/20/2013	ND	--	--	--	--
62	Barnes	ND	B7	3/20/2013	ND	--	--	--	--
63	McIntosh	ND	B8	3/20/2013	ND	--	--	--	--
64	Ottertail	MN	B9	3/20/2013	ND	--	--	--	--
65	Lake of the Woods	MN	B10	3/6/2013	10.472	338	34	0.25	NA[e]
66	Stark	ND	B11	3/6/2013	10.48	350	35	0.26	NA
67	Morton	ND	B12	3/6/2013	10.479	861	79	0.65	NA
68	Logan	ND	B13	3/6/2013	10.483	1192	112	0.90	NA
69	Pembina	ND	B14	3/12/2013	ND	--	--	--	--
70	Roseau	MN	B15	3/20/2013	ND	--	--	--	--
71	Nelson	ND	B16	3/20/2013	ND	--	--	--	--
72	Cavalier	ND	B17	3/20/2013	ND	--	--	--	--
73	Stutsman	ND	B18	3/20/2013	ND	--	--	--	--
74	Ottertail	MN	B19	3/20/2013	ND	--	--	--	--
75	Cavalier	ND	B20	3/20/2013	ND	--	--	--	--
76	Renville	ND	B21	3/20/2013	10.287	655	62	0.48	NA
77	Bottineau	ND	B22	3/20/2013	ND	--	--	--	--
78	Bottineau	ND	B23	3/20/2013	ND	--	--	--	--
79	Towner	ND	B24	3/20/2013	ND	--	--	--	--
80	Burke	ND	B25	3/20/2013	ND	--	--	--	--
81	Towner	ND	B26	3/20/2013	ND	--	--	--	--
82	Cavalier	ND	B27	3/20/2013	ND	--	--	--	--
83	Cavalier	ND	B28	3/20/2013	ND	--	--	--	--
84	Cavalier	ND	B29	3/20/2013	ND	--	--	--	--
85	Renville	ND	B30	3/20/2013	ND	--	--	--	--
86	Rolette	ND	B31	3/20/2013	ND	--	--	--	--
87	Ward	ND	B32	3/6/2013	ND	--	--	--	--
88	Cavalier	ND	B33	3/6/2013	ND	--	--	--	--
89	Rolette	ND	B34	3/6/2013	ND	--	--	--	--
90	Benson	ND	B35	3/6/2013	ND	--	--	--	--
91	Cavalier	ND	B36	3/8/2013	ND	--	--	--	--

92	Burke	ND	B37	3/8/2013	ND	--	--	--	--
93	Burke	ND	B38	3/11/2013	ND	--	--	--	--
94	Mountrail	ND	B39	3/25/2013	ND	--	--	--	--
95	Benson	ND	B40	3/25/2013	ND	--	--	--	--
96	Cavalier	ND	B41	3/25/2013	ND	--	--	--	--
97	Ramsey	ND	B42	3/25/2013	ND	--	--	--	--
98	Pierce	ND	B43	3/25/2013	ND	--	--	--	--
99	Pierce	ND	B44	3/25/2013	ND	--	--	--	--
100	Burke	ND	B45	3/25/2013	ND	--	--	--	--
101	Benson	ND	B46	3/25/2013	10.133	207	22	0.15	NA
102	Towner	ND	B47	3/25/2013	10.136	641	61	0.48	NA
103	Stutsman	ND	B48	3/8/2013	10.158	204	22	0.15	NA
104	Benson	ND	B49	3/8/2013	10.161	295	30	0.21	NA
105	McLean	ND	B50	3/25/2013	10.138	288	29	0.21	NA
106	Pierce	ND	B51	3/25/2013	10.138	283	28	0.21	NA
107	Ward	ND	B52	3/25/2013	ND	--	--	--	--
108	Benson	ND	B53	3/25/2013	ND	--	--	--	--
109	Pennington	MN	B54	3/25/2013	ND	--	--	--	--
110	Cavalier	ND	B55	3/25/2013	ND	--	--	--	--
111	Otter Tail	MN	B56	3/25/2013	10.14	787	75	0.59	NA
112	Pembina	ND	B57	3/25/2013	ND	--	--	--	--
113	Ramsey	ND	B58	3/6/2013	ND	--	--	--	--
114	Stutsman	ND	B59	3/20/2013	ND	--	--	--	--
115	Pembina	ND	B60	3/11/2013	ND	--	--	--	--
116	McHenry	ND	B61	3/11/2013	ND	--	--	--	--
117	Cavalier	ND	B62	3/11/2013	ND	--	--	--	--
118	Burleigh	ND	B63	3/8/2013	ND	--	--	--	--

[a] Retention time of peak
[b] Height of peak
[c] Result of confirmatory test
[d] Not detected
[e] Not applicable as only samples with >1 ng/g were subjected to confirmatory testing

Table A.3. HPLC-FD results for 2011 durum wheat survey samples.

Original Sample #	Julie Sample #	State	Region	HPLC-FD Analysis Date	Ret. Time[a] (min)	Area	Ht.[b]	OTA (ng/g)	Conf.[c]
2181	1	MT	A	8/3/12	ND[d]	--	--	--	--
2201	2	MT	A	4/3/2012	ND	--	--	--	--
2221	3	MT	A	4/26/2012	ND	--	--	--	--
2241	4	MT	A	6/1/2012	ND	--	--	--	--
2261	5	MT	A	7/10/2012	ND	--	--	--	--
2263	6	MT	A	8/2/12	ND	--	--	--	--
2301	7	MT	B	8/3/12	ND	--	--	--	--
2321	8	MT	A	8/3/12	ND	--	--	--	--
2341	9	MT	A	4/11/2012	ND	--	--	--	--
2361	10	MT	B	4/26/2012	ND	--	--	--	--
2363	11	MT	B	6/1/2012	ND	--	--	--	--
2365	12	MT	B	8/2/12	ND	--	--	--	--
2367	13	MT	B	4/26/2012	ND	--	--	--	--
2421	14	MT	B	7/10/2012	ND	--	--	--	--
2441	15	MT	B	8/2/12	ND	--	--	--	--
2461	16	MT	B	6/14/2012	ND	--	--	--	--
2463	17	MT	B	8/2/12	ND	--	--	--	--
2465	18	MT	B	8/2/12	ND	--	--	--	--
2481	19	MT	B	8/2/12	ND	--	--	--	--
2483	20	MT	B	6/13/2012	ND	--	--	--	--
2485	21	MT	B	8/8/12	ND	--	--	--	--
2487	22	MT	B	4/3/2012	ND	--	--	--	--
2489	23	MT	B	8/30/12	ND	--	--	--	--
2491	24	MT	B	4/11/2012	ND	--	--	--	--
2493	25	MT	B	8/8/12	ND	--	--	--	--
2495	26	MT	B	8/3/12	ND	--	--	--	--
2501	27	MT	B	8/2/12	ND	--	--	--	--
2541	28	MT	A	8/2/12	ND	--	--	--	--
4001	29	ND	A	8/2/12	ND	--	--	--	--
4003	30	ND	A	4/4/2012	ND	--	--	--	--
4005	31	ND	A	4/11/2012	ND	--	--	--	--
4007	32	ND	A	8/3/12	ND	--	--	--	--
4009	33	ND	A	7/10/2012	ND	--	--	--	--
4011	34	ND	A	4/12/2012	ND	--	--	--	--
4021	35	ND	A	8/6/12	ND	--	--	--	--
4023	36	ND	A	8/2/12	ND	--	--	--	--
4025	37	ND	A	7/10/2012	ND	--	--	--	--

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Table A.3. HPLC-FD results for 2011 durum wheat survey samples (continued).

4027	38	ND	A	7/10/2012	ND	--	--	--	--
4029	39	ND	A	8/2/12	ND	--	--	--	--
4031	40	ND	A	6/13/2012	ND	--	--	--	--
4041	41	ND	A	4/27/2012	ND	--	--	--	--
4043	42	ND	A	4/3/2012	ND	--	--	--	--
4045	43	ND	A	8/2/12	ND	--	--	--	--
4047	44	ND	A	5/30/2012	ND	--	--	--	--
4049	45	ND	A	7/9/2012	ND	--	--	--	--
4051	46	ND	A	8/8/12	ND	--	--	--	--
4061	47	ND	B	4/26/2012	ND	--	--	--	--
4081	48	ND	B	7/10/2012	ND	--	--	--	--
4083	49	ND	B	4/12/2012	ND	--	--	--	--
4085	50	ND	B	7/10/2012	ND	--	--	--	--
4087	51	ND	B	4/11/2012	10.364	7535	695	5.56	+
4089	52	ND	B	4/12/2012	ND	--	--	--	--
4101	53	ND	A	4/3/2012	ND	--	--	--	--
4103	54	ND	A	7/10/2012	ND	--	--	--	--
4105	55	ND	A	5/15/2012	ND	--	--	--	--
4107	56	ND	A	7/9/2012	ND	--	--	--	--
4109	57	ND	A	8/30/12	ND	--	--	--	--
4111	58	ND	A	8/2/12	ND	--	--	--	--
4121	59	ND	C	4/25/2012	ND	--	--	--	--
4141	60	ND	B	8/2/12	ND	--	--	--	--
4143	61	ND	B	5/15/2012	ND	--	--	--	--
4145	62	ND	B	8/2/12	ND	--	--	--	--
4161	63	ND	B	7/9/2012	ND	--	--	--	--
4181	64	ND	C	4/11/2012	ND	--	--	--	--
4201	65	ND	C	7/9/2012	ND	--	--	--	--
4221	66	ND	C	6/13/2012	ND	--	--	--	--
4223	67	ND	C	6/13/2012	ND	--	--	--	--
4261	68	ND	C	7/10/2012	ND	--	--	--	--
4321	69	ND	C	6/13/2012	ND	--	--	--	--
4341	70	ND	C	4/26/2012	ND	--	--	--	--
4361	71	ND	D	4/25/2012	ND	--	--	--	--
4381	72	ND	A	6/12/2012	ND	--	--	--	--
4383	73	ND	A	4/3/2012	ND	--	--	--	--
4385	74	ND	A	8/2/12	ND	--	--	--	--
4387	75	ND	A	4/25/2012	ND	--	--	--	--
4389	76	ND	A	8/3/12	ND	--	--	--	--
4401	77	ND	B	5/14/2012	ND	--	--	--	--

Table A.3. HPLC-FD results for 2011 durum wheat survey samples (continued).

4403	78	ND	B	4/3/2012	ND	--	--	--	--
4405	79	ND	B	7/10/2012	ND	--	--	--	--
4407	80	ND	B	5/16/2012	ND	--	--	--	--
4409	81	ND	B	7/10/2012	ND	--	--	--	--
4421	82	ND	D	7/10/2012	ND	--	--	--	--
4461	83	ND	C	7/9/2012	ND	--	--	--	--
4481	84	ND	C	4/26/2012	ND	--	--	--	--
4501	85	ND	D	7/9/2012	ND	--	--	--	--
4521	86	ND	B	SNF[e]	--	--	--	--	--
4561	87	ND	C	6/13/2012	ND	--	--	--	--
4681	88	ND	D	7/9/2012	ND	--	--	--	--
4701	89	ND	D	8/3/12	ND	--	--	--	--
4721	90	ND	D	8/2/12	ND	--	--	--	--
4723	91	ND	D	8/3/12	ND	--	--	--	--
4741	92	ND	D	5/30/2012	ND	--	--	--	--
4761	93	ND	D	7/10/2012	ND	--	--	--	--
4763	94	ND	D	6/1/2012	ND	--	--	--	--
4765	95	ND	D	4/3/2012	ND	--	--	--	--
4767	96	ND	D	7/10/2012	ND	--	--	--	--
4781	97	ND	D	8/2/12	ND	--	--	--	--
4783	98	ND	D	6/4/2012	ND	--	--	--	--
4785	99	ND	D	7/10/2012	ND	--	--	--	--
4801	100	ND	D	7/9/2012	ND	--	--	--	--
4803	101	ND	D	5/15/2012	ND	--	--	--	--
4805	102	ND	D	6/1/2012	ND	--	--	--	--
4821	103	ND	D	8/6/12	ND	--	--	--	--
4861	104	ND	D	SNF[e]	--	--	--	--	--
4881	105	ND	D	5/16/12	ND	--	--	--	--

[a] Retention time of peak

[b] Height of peak

[c] Result of confirmatory test

[d] Not detected

[e] Sample not found amongst the survey samples and therefore not analyzed

Table A.4. HPLC-FD results for 2012 durum wheat survey samples.

Original Sample #	Julie Sample #	State	Region	HPLC-FD					
				Analysis Date	Ret. Time[a] (min)	Area	Ht.[b]	OTA (ng/g)	Conf.[c]
2181	D35	MT	A	3/21/2013	10.261	277	29	0.21	NA[d]
2201	D206	MT	A	3/4/2013	ND[e]	--	--	--	--
2202	D12	MT	A	3/11/2013	ND	--	--	--	--
2221	D190	MT	A	3/4/2013	ND	--	--	--	--
2222	D215	MT	A	3/4/2013	ND	--	--	--	--
2241	D213	MT	A	3/4/2013	ND	--	--	--	--
2242	D11	MT	A	3/11/2013	ND	--	--	--	--
2261	D210	MT	A	3/4/2013	ND	--	--	--	--
2262	D51	MT	A	3/11/2013	ND	--	--	--	--
2301	D130	MT	A	3/8/2013	ND	--	--	--	--
2302	D46	MT	A	3/6/2013	ND	--	--	--	--
2321	D24	MT	A	3/6/2013	10.488	262	27	0.20	NA
2322	D40	MT	A	3/21/2013	ND	--	--	--	--
2341	D174	MT	A	3/6/2013	ND	--	--	--	--
2342	D169	MT	A	3/6/2013	ND	--	--	--	--
2361	D216	MT	B	3/4/2013	ND	--	--	--	--
2362	D98	MT	B	3/20/2013	ND	--	--	--	--
2363	D62	MT	B	3/8/2013	ND	--	--	--	--
2364	D71	MT	B	3/12/2013	ND	--	--	--	--
2365	D3	MT	B	3/6/2013	10.473	341	34	0.26	NA
2366	D13	MT	B	3/11/2013	ND	--	--	--	--
2367	D194	MT	B	3/4/2013	ND	--	--	--	--
2368	D20	MT	B	3/11/2013	ND	--	--	--	--
2381	D27	MT	B	3/6/2013	ND	--	--	--	--
2382	D52	MT	B	3/12/2013	ND	--	--	--	--
2421	D165	MT	B	3/6/2013	ND	--	--	--	--
2422	D93	MT	B	3/12/2013	ND	--	--	--	--
2441	D96	MT	B	3/20/2013	ND	--	--	--	--
2442	D182	MT	B	3/6/2013	ND	--	--	--	--
2461	D141	MT	B	3/6/2013	ND	--	--	--	--
2462	D85	MT	B	3/20/2013	10.258	613	59	0.45	NA
2463	D134	MT	B	3/8/2013	ND	--	--	--	--
2464	D65	MT	B	3/8/2013	ND	--	--	--	--
2465	D76	MT	B	3/12/2013	ND	--	--	--	--
2466	D64	MT	B	3/8/2013	ND	--	--	--	--
2467	D66	MT	B	3/8/2013	ND	--	--	--	--
2481	D88	MT	B	3/12/2013	ND	--	--	--	--

Table A.4. HPLC-FD results for 2012 durum wheat survey samples (continued).

2482	D108	MT	B	3/21/2013	ND	--	--	--	--
2483	D162	MT	B	3/6/2013	ND	--	--	--	--
2484	D60	MT	B	3/8/2013	ND	--	--	--	--
2485	D48	MT	B	3/12/2013	ND	--	--	--	--
2486	D124	MT	B	3/8/2013	ND	--	--	--	--
2487	D42	MT	B	3/25/2013	ND	--	--	--	--
2488	D113	MT	B	3/21/2013	ND	--	--	--	--
2489	D16	MT	B	3/11/2013	ND	--	--	--	--
2490	D188	MT	B	3/4/2013	ND	--	--	--	--
2491	D6	MT	B	3/6/2013	10.481	273	28	0.21	NA
2492	D133	MT	B	3/8/2013	ND	--	--	--	--
2493	D54	MT	B	3/12/2013	ND	--	--	--	--
2494	D128	MT	B	3/8/2013	ND	--	--	--	--
2495	D116	MT	B	3/25/2013	ND	--	--	--	--
2501	D2	MT	B	3/6/2013	ND	--	--	--	--
2502	D67	MT	B	3/12/2013	ND	--	--	--	--
2521	D178	MT	B	3/6/2013	ND	--	--	--	--
2522	D212	MT	B	3/4/2013	ND	--	--	--	--
2541	D106	MT	A	3/21/2013	ND	--	--	--	--
2541	D177	MT	B	3/6/2013	ND	--	--	--	--
2542	D202	MT	B	3/6/2013	ND	--	--	--	--
4001	D102	ND	A	3/21/2013	ND	--	--	--	--
4002	D77	ND	A	3/12/2013	ND	--	--	--	--
4003	D92	ND	A	3/12/2013	ND	--	--	--	--
4004	D15	ND	A	3/11/2013	ND	--	--	--	--
4005	D37	ND	A	3/21/2013	ND	--	--	--	--
4006	D172	ND	A	3/6/2013	ND	--	--	--	--
4007	D153	ND	A	3/6/2013	ND	--	--	--	--
4008	D217	ND	A	3/4/2013	ND	--	--	--	--
4009	D140	ND	A	3/8/2013	ND	--	--	--	--
4010	D200	ND	A	3/6/2013	ND	--	--	--	--
4021	D72	ND	A	3/12/2013	ND	--	--	--	--
4022	D176	ND	A	3/6/2013	ND	--	--	--	--
4023	D136	ND	A	3/8/2013	ND	--	--	--	--
4024	D171	ND	A	3/6/2013	ND	--	--	--	--
4025	D219	ND	A	3/4/2013	10.459	511	50	0.37	NA
4026	D187	ND	A	3/4/2013	ND	--	--	--	--
4027	D122	ND	A	3/8/2013	ND	--	--	--	--
4028	D159	ND	A	3/6/2013	ND	--	--	--	--
4029	D220	ND	A	3/4/2013	ND	--	--	--	--

4030	D183	ND	A	3/4/2013	ND	--	--	--	--
4031	D170	ND	A	3/6/2013	ND	--	--	--	--
4032	D154	ND	A	3/6/2013	ND	--	--	--	--
4033	D185	ND	A	3/4/2013	ND	--	--	--	--
4034	D164	ND	A	3/6/2013	ND	--	--	--	--
4035	D156	ND	A	3/6/2013	ND	--	--	--	--
4040	D55	ND	B	3/11/2013	ND	--	--	--	--
4041	D105	ND	A	3/21/2013	ND	--	--	--	--
4042	D126	ND	A	3/8/2013	ND	--	--	--	--
4043	D19	ND	A	3/11/2013	ND	--	--	--	--
4044	D30	ND	A	3/6/2013	ND	--	--	--	--
4045	D59	ND	A	3/8/2013	ND	--	--	--	--
4046	D47	ND	A	3/11/2013	ND	--	--	--	--
4047	D90	ND	A	3/12/2013	ND	--	--	--	--
4048	D91	ND	A	3/12/2013	ND	--	--	--	--
4049	D80	ND	A	3/20/2013	ND	--	--	--	--
4050	D5	ND	A	3/6/2013	ND	--	--	--	--
4051	D10	ND	A	3/11/2013	ND	--	--	--	--
4052	D22	ND	A	3/11/2013	ND	--	--	--	--
4053	D104	ND	A	3/21/2013	10.279	242	25	0.18	NA
4054	D214	ND	A	3/4/2013	10.464	612	60	0.45	NA
4081	D4	ND	B	3/20/2013	10.254	376	38	0.28	NA
4082	D49	ND	B	3/11/2013	ND	--	--	--	--
4083	D50	ND	B	3/12/2013	ND	--	--	--	--
4084	D111	ND	B	3/21/2013	ND	--	--	--	--
4085	D21	ND	B	3/11/2013	ND	--	--	--	--
4086	D94	ND	B	3/20/2013	ND	--	--	--	--
4087	D180	ND	B	3/6/2013	ND	--	--	--	--
4088	D39	ND	B	3/21/2013	ND	--	--	--	--
4089	D45	ND	B	3/25/2013	ND	--	--	--	--
4091	D147	ND	B	3/6/2013	ND	--	--	--	--
4093	D151	ND	B	3/6/2013	ND	--	--	--	--
4094	D175	ND	B	3/6/2013	ND	--	--	--	--
4095	D160	ND	B	3/6/2013	ND	--	--	--	--
4095	D205	ND	B	3/4/2013	ND	--	--	--	--
4096	D137	ND	B	3/8/2013	ND	--	--	--	--
4101	D75	ND	A	3/12/2013	ND	--	--	--	--
4102	D81	ND	A	3/20/2013	ND	--	--	--	--
4102	D107	ND	B	3/21/2013	ND	--	--	--	--
4103	D110	ND	A	3/21/2013	ND	--	--	--	--

Table A.4. HPLC-FD results for 2012 durum wheat survey samples (continued).

4104	D201	ND	A	3/6/2013	ND	--	--	--	--
4105	D101	ND	A	3/20/2013	ND	--	--	--	--
4106	D148	ND	A	3/6/2013	ND	--	--	--	--
4107	D207	ND	A	3/4/2013	10.46	578	56	0.42	NA
4108	D198	ND	A	3/6/2013	ND	--	--	--	--
4109	D120	ND	A	3/25/2013	ND	--	--	--	--
4110	D119	ND	A	3/25/2013	ND	--	--	--	--
4111	D157	ND	A	3/6/2013	ND	--	--	--	--
4113	D208	ND	A	3/4/2013	ND	--	--	--	--
4114	D131	ND	A	3/8/2013	ND	--	--	--	--
4115	D152	ND	A	3/6/2013	ND	--	--	--	--
4141	D1	ND	B	3/6/2013	ND	--	--	--	--
4142	D184	ND	B	3/4/2013	ND	--	--	--	--
4143	D197	ND	B	3/6/2013	ND	--	--	--	--
4144	D118	ND	B	3/25/2013	ND	--	--	--	--
4145	D123	ND	B	3/8/2013	ND	--	--	--	--
4161	D117	ND	B	3/21/2013	ND	--	--	--	--
4163	D163	ND	B	3/6/2013	ND	--	--	--	--
4164	D69	ND	B	3/12/2013	ND	--	--	--	--
4165	D218	ND	B	3/4/2013	10.458	264	27	0.19	NA
4166	D166	ND	B	3/6/2013	ND	--	--	--	--
4181	D179	ND	C	3/6/2013	ND	--	--	--	--
4201	D58	ND	C	3/12/2013	ND	--	--	--	--
4202	D68	ND	C	3/12/2013	ND	--	--	--	--
4221	D7	ND	C	3/20/2013	ND	--	--	--	--
4222	D36	ND	C	3/21/2013	ND	--	--	--	--
4223	D97	ND	C	3/20/2013	ND	--	--	--	--
4261	D82	ND	C	3/20/2013	ND	--	--	--	--
4301	D61	ND	C	3/8/2013	ND	--	--	--	--
4302	D161	ND	C	3/6/2013	ND	--	--	--	--
4321	D167	ND	C	3/6/2013	ND	--	--	--	--
4322	D193	ND	C	3/4/2013	ND	--	--	--	--
4361	D70	ND	D	3/12/2013	ND	--	--	--	--
4362	D139	ND	D	3/8/2013	ND	--	--	--	--
4381	D132	ND	A	3/8/2013	ND	--	--	--	--
4382	D211	ND	A	3/4/2013	ND	--	--	--	--
4383	D112	ND	A	3/21/2013	ND	--	--	--	--
4384	D145	ND	A	3/6/2013	ND	--	--	--	--
4385	D158	ND	A	3/6/2013	ND	--	--	--	--
4386	D146	ND	A	3/6/2013	ND	--	--	--	--

Table A.4. HPLC-FD results for 2012 durum wheat survey samples (continued).

4387	D209	ND	A	3/4/2013	ND	--	--	--	--
4388	D149	ND	A	3/6/2013	ND	--	--	--	--
4389	D186	ND	A	3/4/2013	ND	--	--	--	--
4390	D129	ND	A	3/8/2013	ND	--	--	--	--
4391	D199	ND	A	3/6/2013	ND	--	--	--	--
4392	D196	ND	A	3/4/2013	10.471	776	76	0.57	NA
4401	D41	ND	B	3/25/2013	ND	--	--	--	--
4402	D73	ND	B	3/12/2013	ND	--	--	--	--
4403	D44	ND	B	3/25/2013	ND	--	--	--	--
4404	D121	ND	B	3/8/2013	ND	--	--	--	--
4405	D38	ND	B	3/21/2013	10.268	12190	1139	9.11	+
4406	D53	ND	B	3/12/2013	ND	--	--	--	--
4407	D173	ND	B	3/6/2013	ND	--	--	--	--
4408	D100	ND	B	3/20/2013	ND	--	--	--	--
4409	D23	ND	B	3/6/2013	ND	--	--	--	--
4410	D181	ND	B	3/6/2013	ND	--	--	--	--
4411	D25	ND	B	3/11/2013	ND	--	--	--	--
4412	D57	ND	B	3/8/2013	ND	--	--	--	--
4413	D56	ND	B	3/11/2013	ND	--	--	--	--
4414	D189	ND	B	3/4/2013	10.463	639	61	0.47	NA
4415	D8	ND	B	3/20/2013	10.257	248	26	0.18	NA
4421	D17	ND	D	3/11/2013	ND	--	--	--	--
4422	D168	ND	D	3/6/2013	ND	--	--	--	--
4423	D115	ND	D	3/21/2013	ND	--	--	--	--
4521	D89	ND	B	3/20/2013	10.26	658	63	0.49	NA
4681	D87	ND	D	3/20/2013	ND	--	--	--	--
4682	D95	ND	D	3/20/2013	ND	--	--	--	--
4683	D103	ND	D	3/21/2013	ND	--	--	--	--
4701	D99	ND	D	3/20/2013	10.258	226	24	0.17	NA
4702	D43	ND	D	3/25/2013	ND	--	--	--	--
4721	D29	ND	D	3/6/2013	ND	--	--	--	--
4721	D84	ND	D	3/20/2013	ND	--	--	--	--
4723	D26	ND	D	3/11/2013	ND	--	--	--	--
4724	D14	ND	D	3/11/2013	ND	--	--	--	--
4725	D142	ND	D	3/8/2013	ND	--	--	--	--
4726	D9	ND	D	3/8/2013	ND	--	--	--	--
4741	D83	ND	D	3/20/2013	ND	--	--	--	--
4742	D155	ND	D	3/6/2013	ND	--	--	--	--
4743	D63	ND	D	3/8/2013	ND	--	--	--	--
4761	D18	ND	D	3/11/2013	ND	--	--	--	--

4762	D109	ND	D	3/21/2013	ND	--	--	--	--
4763	D79	ND	D	3/20/2013	ND	--	--	--	--
4764	D192	ND	D	3/4/2013	ND	--	--	--	--
4765	D204	ND	D	3/6/2013	ND	--	--	--	--
4766	D34	ND	D	3/6/2013	10.478	8991	826	6.78	+
4767	D86	ND	D	3/20/2013	ND	--	--	--	--
4768	D114	ND	D	3/21/2013	ND	--	--	--	--
4781	D74	ND	D	3/12/2013	ND	--	--	--	--
4782	D31	ND	D	3/6/2013	ND	--	--	--	--
4783	D191	ND	D	3/4/2013	ND	--	--	--	--
4784	D195	ND	D	3/4/2013	ND	--	--	--	--
4785	D203	ND	D	3/6/2013	ND	--	--	--	--
4801	D125	ND	D	3/8/2013	ND	--	--	--	--
4802	D144	ND	D	3/21/2013	ND	--	--	--	--
4803	D33	ND	D	3/6/2013	ND	--	--	--	--
4804	D150	ND	D	3/6/2013	ND	--	--	--	--
4805	D78	ND	D	3/12/2013	ND	--	--	--	--
4806	D32	ND	D	3/6/2013	ND	--	--	--	--
4821	D127	ND	D	3/8/2013	ND	--	--	--	--
4861	D143	ND	D	3/21/2013	ND	--	--	--	--
4862	D28	ND	D	3/6/2013	ND	--	--	--	--
4881	D138	ND	D	3/8/2013	ND	--	--	--	--
4882	D135	ND	D	3/8/2013	ND	--	--	--	--

[a] Retention time of peak

[b] Height of peak

[c] Result of confirmatory test

[d] Not applicable as only samples with >1 ng/g were subjected to confirmatory testing

[e] Not detected

Table A.5. HPLC-FD results for 2011 hard red spring wheat survey samples.

| Original Sample # | Julie Sample # | State | Region | HPLC-FD | | | | | |
				Analysis Date	Ret. Time[a] (min)	Area	Ht.[b]	OTA (ng/g)	Conf.[c]
1	1	MN	B	8/30/12	ND[d]	--	--	--	--
2	2	MN	B	8/29/12	ND	--	--	--	--
3	3	MN	B	5/30/2012	ND	--	--	--	--
21	4	MN	B	8/20/12	ND	--	--	--	--
22	5	MN	B	7/10/2012	ND	--	--	--	--
23	6	MN	B	6/11/2012	ND	--	--	--	--
24	7	MN	B	8/28/12	ND	--	--	--	--
25	8	MN	B	8/28/12	ND	--	--	--	--
26	9	MN	B	6/12/2012	ND	--	--	--	--
61	10	MN	A	8/24/12	ND	--	--	--	--
62	11	MN	A	5/30/2012	ND	--	--	--	--
63	12	MN	A	6/13/2012	ND	--	--	--	--
64	13	MN	A	4/12/2012	ND	--	--	--	--
65	14	MN	A	6/13/2012	ND	--	--	--	--
66	15	MN	A	4/25/2012	ND	--	--	--	--
67	16	MN	A	6/11/2012	ND	--	--	--	--
81	17	MN	A	8/28/12	ND	--	--	--	--
101	18	MN	A	5/30/2012	ND	--	--	--	--
102	19	MN	A	5/14/2012	ND	--	--	--	--
103	20	MN	A	6/6/12	ND	--	--	--	--
104	21	MN	A	8/27/12	ND	--	--	--	--
105	22	MN	A	5/30/2012	ND	--	--	--	--
106	23	MN	A	8/27/2012	ND	--	--	--	--
107	24	MN	A	5/30/2012	ND	--	--	--	--
108	25	MN	A	8/29/12	ND	--	--	--	--
109	26	MN	A	5/30/2012	ND	--	--	--	--
121	27	MN	A	6/4/2012	ND	--	--	--	--
122	28	MN	A	8/30/12	ND	--	--	--	--
123	29	MN	A	8/29/12	ND	--	--	--	--
124	30	MN	A	7/6/2012	ND	--	--	--	--
125	31	MN	A	6/4/2012	ND	--	--	--	--
126	32	MN	A	8/8/12	ND	--	--	--	--
127	33	MN	A	8/3/12	ND	--	--	--	--
141	34	MN	A	7/6/2012	ND	--	--	--	--
142	35	MN	A	8/8/12	ND	--	--	--	--
143	36	MN	A	6/6/12	ND	--	--	--	--

Table A.5. HPLC-FD results for 2011 hard red spring wheat survey samples (continued).

144	37	MN	A	6/1/2012	ND	--	--	--	--
145	38	MN	A	8/27/2012	ND	--	--	--	--
161	39	MN	A	8/27/2012	ND	--	--	--	--
162	40	MN	A	8/21/12	ND	--	--	--	--
163	41	MN	A	8/20/12	ND	--	--	--	--
164	42	MN	A	6/4/2012	ND	--	--	--	--
165	43	MN	A	6/11/2012	ND	--	--	--	--
166	44	MN	A	8/28/12	ND	--	--	--	--
167	45	MN	A	6/11/2012	ND	--	--	--	--
168	46	MN	A	6/11/2012	ND	--	--	--	--
169	47	MN	A	4/12/2012	ND	--	--	--	--
181	48	MN	A	5/15/2012	ND	--	--	--	--
182	49	MN	A	5/15/2012	ND	--	--	--	--
183	50	MN	A	8/29/12	ND	--	--	--	--
184	51	MN	A	6/4/2012	ND	--	--	--	--
201	52	MN	A	7/5/2012	ND	--	--	--	--
202	53	MN	A	8/8/12	ND	--	--	--	--
203	54	MN	A	6/1/2012	ND	--	--	--	--
204	55	MN	A	8/8/12	ND	--	--	--	--
205	56	MN	A	8/29/12	ND	--	--	--	--
321	57	MN	A	8/3/12	ND	--	--	--	--
461	58	MN	B	8/8/12	ND	--	--	--	--
462	59	MN	B	8/24/2012	ND	--	--	--	--
501	60	MN	B	7/3/2012	ND	--	--	--	--
502	61	MN	B	6/14/2012	ND	--	--	--	--
503	62	MN	B	8/3/12	ND	--	--	--	--
504	63	MN	B	7/10/2012	ND	--	--	--	--
581	64	MN	B	5/14/2012	ND	--	--	--	--
582	65	MN	B	6/14/2012	ND	--	--	--	--
601	66	MN	B	7/9/2012	ND	--	--	--	--
602	67	MN	B	6/12/2012	ND	--	--	--	--
603	68	MN	B	6/6/12	ND	--	--	--	--
604	69	MN	B	6/14/2012	ND	--	--	--	--
605	70	MN	B	6/6/12	ND	--	--	--	--
606	71	MN	B	5/15/2012	ND	--	--	--	--
607	72	MN	B	8/24/2012	ND	--	--	--	--
2181	73	MT	A	6/1/2012	ND	--	--	--	--
2182	74	MT	A	6/14/2012	ND	--	--	--	--
2183	75	MT	A	6/1/2012	ND	--	--	--	--
2184	76	MT	A	6/14/2012	ND	--	--	--	--

Table A.5. HPLC-FD results for 2011 hard red spring wheat survey samples (continued).

2201	77	MT	A	5/30/2012	ND	--	--	--	--
2202	78	MT	A	8/27/2012	ND	--	--	--	--
2203	79	MT	A	8/6/12	ND	--	--	--	--
2221	80	MT	A	8/2/12	ND	--	--	--	--
2222	81	MT	A	6/4/2012	ND	--	--	--	--
2223	82	MT	A	4/11/2012	ND	--	--	--	--
2224	83	MT	A	6/14/2012	ND	--	--	--	--
2225	84	MT	A	8/21/12	ND	--	--	--	--
2241	85	MT	A	8/6/12	ND	--	--	--	--
2242	86	MT	A	8/6/12	ND	--	--	--	--
2243	87	MT	A	6/1/2012	ND	--	--	--	--
2244	88	MT	A	6/14/2012	ND	--	--	--	--
2245	89	MT	A	8/3/12	ND	--	--	--	--
2261	90	MT	A	8/20/12	ND	--	--	--	--
2262	91	MT	A	7/3/2012	ND	--	--	--	--
2281	92	MT	A	8/6/12	ND	--	--	--	--
2282	93	MT	A	8/3/12	ND	--	--	--	--
2283	94	MT	A	8/3/12	ND	--	--	--	--
2284	95	MT	A	8/8/12	ND	--	--	--	--
2301	96	MT	A	6/1/2012	ND	--	--	--	--
2302	97	MT	A	6/1/2012	ND	--	--	--	--
2303	98	MT	A	4/12/2012	ND	--	--	--	--
2304	99	MT	A	8/2/12	ND	--	--	--	--
2321	100	MT	A	7/10/2012	ND	--	--	--	--
2322	101	MT	A	8/6/12	ND	--	--	--	--
2341	102	MT	A	7/9/2012	ND	--	--	--	--
2342	103	MT	A	8/30/12	ND	--	--	--	--
2343	104	MT	A	8/24/2012	ND	--	--	--	--
2344	105	MT	A	8/20/12	ND	--	--	--	--
2345	106	MT	A	6/11/2012	ND	--	--	--	--
2346	107	MT	A	8/20/12	ND	--	--	--	--
2361	108	MT	B	8/24/2012	ND	--	--	--	--
2362	109	MT	B	8/29/12	ND	--	--	--	--
2363	110	MT	B	6/4/2012	ND	--	--	--	--
2364	111	MT	B	6/14/2012	ND	--	--	--	--
2365	112	MT	B	7/6/2012	ND	--	--	--	--
2381	113	MT	B	5/17/2012	ND	--	--	--	--
2382	114	MT	B	6/1/2012	ND	--	--	--	--
2383	115	MT	B	6/1/2012	ND	--	--	--	--
2384	116	MT	B	8/20/12	ND	--	--	--	--

Table A.5. HPLC-FD results for 2011 hard red spring wheat survey samples (continued).

2401	117	MT	B	5/30/2012	ND	--	--	--	--
2402	118	MT	B	8/24/2012	ND	--	--	--	--
2421	119	MT	B	6/14/2012	ND	--	--	--	--
2422	120	MT	B	6/6/12	ND	--	--	--	--
2423	121	MT	B	6/11/2012	ND	--	--	--	--
2441	122	MT	B	7/6/2012	ND	--	--	--	--
2442	123	MT	B	8/20/12	ND	--	--	--	--
2443	124	MT	B	8/3/12	ND	--	--	--	--
2444	125	MT	B	8/2/12	ND	--	--	--	--
2445	126	MT	B	8/8/12	ND	--	--	--	--
2461	127	MT	B	6/14/2012	ND	--	--	--	--
2462	128	MT	B	7/6/2012	ND	--	--	--	--
2463	129	MT	B	5/30/2012	ND	--	--	--	--
2464	130	MT	B	5/17/2012	ND	--	--	--	--
2465	131	MT	B	5/17/2012	ND	--	--	--	--
2466	132	MT	B	7/9/2012	ND	--	--	--	--
2481	133	MT	B	8/8/12	ND	--	--	--	--
2501	134	MT	B	6/13/2012	ND	--	--	--	--
2502	135	MT	B	6/4/2012	ND	--	--	--	--
2503	136	MT	B	8/6/12	ND	--	--	--	--
2504	137	MT	B	6/12/2012	ND	--	--	--	--
2505	138	MT	B	8/6/12	ND	--	--	--	--
2506	139	MT	B	6/1/2012	ND	--	--	--	--
2507	140	MT	B	6/11/2012	ND	--	--	--	--
2508	141	MT	B	8/6/12	ND	--	--	--	--
2521	142	MT	C	6/12/2012	ND	--	--	--	--
2522	143	MT	C	5/30/2012	ND	--	--	--	--
2541	144	MT	C	8/28/12	ND	--	--	--	--
2561	145	MT	C	8/3/12	ND	--	--	--	--
2562	146	MT	C	6/11/2012	ND	--	--	--	--
2721	147	MT	E	8/29/12	ND	--	--	--	--
2741	148	MT	E	8/6/12	ND	--	--	--	--
2742	149	MT	E	8/6/12	ND	--	--	--	--
2781	150	MT	E	8/8/12	ND	--	--	--	--
3001	151	MT	D	6/4/2012	ND	--	--	--	--
3041	152	MT	D	6/1/2012	ND	--	--	--	--
3061	153	MT	D	6/6/12	ND	--	--	--	--
3081	154	MT	D	7/9/2012	ND	--	--	--	--
4001	155	ND	A	8/24/12	ND	--	--	--	--
4002	156	ND	A	5/14/2012	ND	--	--	--	--

Table A.5. HPLC-FD results for 2011 hard red spring wheat survey samples (continued).

4003	157	ND	A	8/3/12	ND	--	--	--	--
4004	158	ND	A	7/3/2012	ND	--	--	--	--
4021	159	ND	A	6/11/2012	ND	--	--	--	--
4041	160	ND	A	8/8/12	ND	--	--	--	--
4042	161	ND	A	6/1/2012	ND	--	--	--	--
4043	162	ND	A	5/15/2012	ND	--	--	--	--
4061	163	ND	A	6/11/2012	ND	--	--	--	--
4062	164	ND	A	8/29/12	ND	--	--	--	--
4063	165	ND	A	7/6/2012	ND	--	--	--	--
4081	166	ND	A	8/29/12	ND	--	--	--	--
4082	167	ND	A	8/27/12	ND	--	--	--	--
4083	168	ND	A	6/4/2012	ND	--	--	--	--
4084	169	ND	A	6/11/2012	ND	--	--	--	--
4085	170	ND	A	6/13/2012	ND	--	--	--	--
4086	171	ND	A	8/28/12	ND	--	--	--	--
4087	172	ND	A	6/14/2012	ND	--	--	--	--
4088	173	ND	A	8/24/12	ND	--	--	--	--
4089	174	ND	A	7/6/2012	ND	--	--	--	--
4101	175	ND	A	8/24/2012	ND	--	--	--	--
4102	176	ND	A	5/15/2012	ND	--	--	--	--
4103	177	ND	A	4/11/2012	ND	--	--	--	--
4121	178	ND	B	5/17/2012	ND	--	--	--	--
4122	179	ND	B	6/11/2012	ND	--	--	--	--
4123	180	ND	B	8/30/12	ND	--	--	--	--
4124	181	ND	B	7/3/2012	ND	--	--	--	--
4125	182	ND	B	8/6/12	ND	--	--	--	--
4141	183	ND	A	6/4/2012	ND	--	--	--	--
4142	184	ND	A	8/28/12	ND	--	--	--	--
4143	185	ND	A	6/1/2012	ND	--	--	--	--
4144	186	ND	A	6/6/12	ND	--	--	--	--
4145	187	ND	A	6/1/2012	ND	--	--	--	--
4146	188	ND	A	5/17/2012	ND	--	--	--	--
4147	189	ND	A	8/29/12	ND	--	--	--	--
4148	190	ND	A	8/27/12	ND	--	--	--	--
4161	191	ND	A	5/15/2012	ND	--	--	--	--
4162	192	ND	A	8/21/12	ND	--	--	--	--
4163	193	ND	A	8/6/12	ND	--	--	--	--
4164	194	ND	A	--[e]	ND	--	--	--	--
4165	195	ND	A	6/4/2012	ND	--	--	--	--
4181	196	ND	B	6/1/2012	ND	--	--	--	--

Table A.5. HPLC-FD results for 2011 hard red spring wheat survey samples (continued).

4182	197	ND	B	6/4/2012	ND	--	--	--	--
4183	198	ND	B	8/24/2012	ND	--	--	--	--
4184	199	ND	B	8/24/12	ND	--	--	--	--
4185	200	ND	B	8/27/2012	ND	--	--	--	--
4201	201	ND	B	5/30/2012	ND	--	--	--	--
4202	202	ND	B	8/29/12	ND	--	--	--	--
4203	203	ND	B	8/29/12	ND	--	--	--	--
4204	204	ND	B	8/28/12	ND	--	--	--	--
4221	205	ND	B	8/27/2012	ND	--	--	--	--
4222	206	ND	B	8/21/12	ND	--	--	--	--
4223	207	ND	B	8/8/12	ND	--	--	--	--
4224	208	ND	B	--	ND	--	--	--	--
4225	209	ND	B	8/2/12	ND	--	--	--	--
4226	210	ND	B	8/6/12	ND	--	--	--	--
4227	211	ND	B	7/6/2012	ND	--	--	--	--
4228	212	ND	B	6/4/2012	ND	--	--	--	--
4229	213	ND	B	8/6/12	ND	--	--	--	--
4241	214	ND	C	8/24/12	ND	--	--	--	--
4242	215	ND	C	8/21/12	ND	--	--	--	--
4243	216	ND	C	8/21/12	ND	--	--	--	--
4244	217	ND	C	8/8/12	ND	--	--	--	--
4245	218	ND	C	8/8/12	ND	--	--	--	--
4246	219	ND	C	7/9/2012	ND	--	--	--	--
4247	220	ND	C	6/12/2012	ND	--	--	--	--
4248	221	ND	C	6/4/2012	ND	--	--	--	--
4249	222	ND	C	5/14/2012	ND	--	--	--	--
4261	223	ND	B	8/24/12	ND	--	--	--	--
4262	224	ND	B	6/14/2012	ND	--	--	--	--
4263	225	ND	B	7/3/2012	ND	--	--	--	--
4264	226	ND	B	8/21/12	ND	--	--	--	--
4265	227	ND	B	8/24/2012	ND	--	--	--	--
4281	228	ND	C	8/28/12	ND	--	--	--	--
4282	229	ND	C	6/4/2012	ND	--	--	--	--
4283	230	ND	C	6/6/12	ND	--	--	--	--
4284	231	ND	C	6/4/2012	ND	--	--	--	--
4285	232	ND	C	5/16/2012	ND	--	--	--	--
4286	233	ND	C	7/9/2012	ND	--	--	--	--
4287	234	ND	C	8/20/12	ND	--	--	--	--
4288	235	ND	C	8/24/12	ND	--	--	--	--
4289	236	ND	C	8/3/12	ND	--	--	--	--

Table A.5. HPLC-FD results for 2011 hard red spring wheat survey samples (continued).

4301	237	ND	B	7/6/2012	ND	--	--	--	--
4302	238	ND	B	6/14/2012	ND	--	--	--	--
4303	239	ND	B	8/24/12	ND	--	--	--	--
4304	240	ND	B	8/3/12	ND	--	--	--	--
4305	241	ND	B	6/4/2012	ND	--	--	--	--
4321	242	ND	B	5/15/2012	ND	--	--	--	--
4322	243	ND	B	6/1/2012	ND	--	--	--	--
4323	244	ND	B	8/27/12	ND	--	--	--	--
4324	245	ND	B	6/11/2012	ND	--	--	--	--
4325	246	ND	B	6/13/2012	ND	--	--	--	--
4326	247	ND	B	6/11/2012	ND	--	--	--	--
4327	248	ND	B	8/6/12	ND	--	--	--	--
4328	249	ND	B	6/14/2012	ND	--	--	--	--
4341	250	ND	C	5/15/2012	ND	--	--	--	--
4342	251	ND	C	6/14/2012	ND	--	--	--	--
4343	252	ND	C	5/15/2012	ND	--	--	--	--
4344	253	ND	C	8/28/12	ND	--	--	--	--
4345	254	ND	C	8/27/12	ND	--	--	--	--
4346	255	ND	C	5/30/2012	ND	--	--	--	--
4347	256	ND	C	5/30/2012	ND	--	--	--	--
4348	257	ND	C	4/12/2012	ND	--	--	--	--
4349	258	ND	C	6/1/2012	ND	--	--	--	--
4361	259	ND	D	6/6/12	ND	--	--	--	--
4362	260	ND	D	6/12/2012	ND	--	--	--	--
4363	261	ND	D	8/29/12	ND	--	--	--	--
4381	262	ND	A	8/30/12	ND	--	--	--	--
4382	263	ND	A	6/4/2012	ND	--	--	--	--
4401	264	ND	A	5/14/2012	ND	--	--	--	--
4402	265	ND	A	6/1/2012	ND	--	--	--	--
4403	266	ND	A	8/20/12	ND	--	--	--	--
4404	267	ND	A	8/21/12	ND	--	--	--	--
4405	268	ND	A	8/27/2012	ND	--	--	--	--
4406	269	ND	A	8/29/12	ND	--	--	--	--
4407	270	ND	A	8/30/12	ND	--	--	--	--
4408	271	ND	A	8/28/12	ND	--	--	--	--
4409	272	ND	A	6/1/2012	ND	--	--	--	--
4421	273	ND	D	8/21/12	ND	--	--	--	--
4422	274	ND	D	8/6/12	ND	--	--	--	--
4441	275	ND	D	6/11/2012	ND	--	--	--	--
4442	276	ND	D	6/12/2012	ND	--	--	--	--

Table A.5. HPLC-FD results for 2011 hard red spring wheat survey samples (continued).

4443	277	ND	D	8/24/2012	ND	--	--	--	--
4461	278	ND	B	6/4/2012	ND	--	--	--	--
4462	279	ND	B	5/17/2012	ND	--	--	--	--
4481	280	ND	B	8/21/12	ND	--	--	--	--
4482	281	ND	B	5/16/2012	ND	--	--	--	--
4483	282	ND	B	8/24/2012	ND	--	--	--	--
4501	283	ND	E	8/27/12	ND	--	--	--	--
4502	284	ND	E	8/29/12	ND	--	--	--	--
4521	285	ND	A	6/14/2012	ND	--	--	--	--
4522	286	ND	A	7/10/2012	ND	--	--	--	--
4523	287	ND	A	5/17/2012	ND	--	--	--	--
4541	288	ND	E	5/14/2012	ND	--	--	--	--
4542	289	ND	E	8/20/12	ND	--	--	--	--
4543	290	ND	E	8/20/12	ND	--	--	--	--
4544	291	ND	E	8/20/12	ND	--	--	--	--
4545	292	ND	E	8/29/12	ND	--	--	--	--
4546	293	ND	E	6/11/2012	ND	--	--	--	--
4561	294	ND	B	6/11/2012	ND	--	--	--	--
4562	295	ND	B	5/17/2012	ND	--	--	--	--
4563	296	ND	B	8/21/12	ND	--	--	--	--
4564	297	ND	B	5/15/2012	ND	--	--	--	--
4565	298	ND	B	5/15/2012	ND	--	--	--	--
4566	299	ND	B	5/15/2012	ND	--	--	--	--
4567	300	ND	B	8/3/12	ND	--	--	--	--
4568	301	ND	B	8/6/12	ND	--	--	--	--
4581	302	ND	F	8/8/12	ND	--	--	--	--
4582	303	ND	F	8/8/12	ND	--	--	--	--
4583	304	ND	F	7/3/2012	ND	--	--	--	--
4584	305	ND	F	5/30/2012	ND	--	--	--	--
4585	306	ND	F	8/27/12	ND	--	--	--	--
4586	307	ND	F	8/21/12	ND	--	--	--	--
4601	308	ND	F	6/14/2012	ND	--	--	--	--
4602	309	ND	F	7/6/2012	ND	--	--	--	--
4603	310	ND	F	7/9/2012	ND	--	--	--	--
4604	311	ND	F	8/28/12	ND	--	--	--	--
4605	312	ND	F	8/27/12	ND	--	--	--	--
4606	313	ND	F	8/27/2012	ND	--	--	--	--
4607	314	ND	F	6/12/2012	ND	--	--	--	--
4621	315	ND	B	7/6/2012	ND	--	--	--	--
4622	316	ND	B	7/3/2012	ND	--	--	--	--

Table A.5. HPLC-FD results for 2011 hard red spring wheat survey samples (continued).

4623	317	ND	B	6/12/2012	ND	--	--	--	--
4641	318	ND	C	5/15/2012	ND	--	--	--	--
4642	319	ND	C	6/13/2012	ND	--	--	--	--
4643	320	ND	C	8/3/12	ND	--	--	--	--
4661	321	ND	C	8/8/12	ND	--	--	--	--
4662	322	ND	C	8/21/12	ND	--	--	--	--
4663	323	ND	C	8/28/12	ND	--	--	--	--
4664	324	ND	C	5/30/2012	ND	--	--	--	--
4665	325	ND	C	8/28/12	ND	--	--	--	--
4681	326	ND	D	6/4/2012	ND	--	--	--	--
4682	327	ND	D	6/1/2012	ND	--	--	--	--
4683	328	ND	D	6/11/2012	ND	--	--	--	--
4701	329	ND	D	8/24/12	ND	--	--	--	--
4721	330	ND	D	5/30/2012	ND	--	--	--	--
4722	331	ND	D	8/2/12	ND	--	--	--	--
4741	332	ND	D	7/6/2012	ND	--	--	--	--
4761	333	ND	D	6/6/12	ND	--	--	--	--
4762	334	ND	D	7/3/2012	ND	--	--	--	--
4763	335	ND	D	8/27/12	ND	--	--	--	--
4764	336	ND	D	8/21/12	ND	--	--	--	--
4765	337	ND	D	8/2/12	ND	--	--	--	--
4766	338	ND	D	4/26/2012	ND	-	-	-	--
4767	339	ND	D	8/6/12	ND	--	--	--	--
4768	340	ND	D	8/6/12	ND	--	--	--	--
4781	341	ND	D	8/27/12	ND	--	--	--	--
4782	342	ND	D	8/27/12	ND	--	--	--	--
4783	343	ND	D	5/30/2012	ND	--	--	--	--
4801	344	ND	D	5/16/2012	ND	--	--	--	--
4802	345	ND	D	5/17/2012	ND	--	--	--	--
4803	346	ND	D	8/20/12	ND	--	--	--	--
4804	347	ND	D	8/30/12	ND	--	--	--	--
4805	348	ND	D	5/17/2012	ND	--	--	--	--
4806	349	ND	D	8/30/12	ND	--	--	--	--
4807	350	ND	D	8/3/12	ND	--	--	--	--
4821	351	ND	E	5/15/2012	ND	--	--	--	--
4822	352	ND	E	8/28/12	ND	--	--	--	--
4823	353	ND	E	8/29/12	ND	--	--	--	--
4824	354	ND	E	5/17/2012	ND	--	--	--	--
4841	355	ND	E	5/15/2012	ND	--	--	--	--
4842	356	ND	E	8/21/12	ND	--	--	--	--

Table A.5. HPLC-FD results for 2011 hard red spring wheat survey samples (continued).

4843	357	ND	E	6/11/2012	ND	--	--	--	--
4844	358	ND	E	5/16/2012	ND	--	--	--	--
4861	359	ND	D	8/30/12	ND	--	--	--	--
4862	360	ND	D	8/30/12	ND	--	--	--	--
4863	361	ND	D	8/27/12	ND	--	--	--	--
4864	362	ND	D	8/24/12	ND	--	--	--	--
4881	363	ND	D	6/6/12	ND	--	--	--	--
4882	364	ND	D	6/11/2012	ND	--	--	--	--
4883	365	ND	D	5/15/2012	ND	--	--	--	--
4884	366	ND	D	5/14/2012	ND	--	--	--	--
4885	367	ND	D	6/4/2012	ND	--	--	--	--
4901	368	ND	D	6/14/2012	ND	--	--	--	--
4921	369	ND	E	8/27/12	ND	--	--	--	--
4941	370	ND	E	8/21/12	ND	--	--	--	--
4942	371	ND	E	8/28/12	ND	--	--	--	--
4943	372	ND	E	8/28/12	ND	--	--	--	--
4961	373	ND	E	8/28/12	ND	--	--	--	--
4962	374	ND	E	8/28/12	ND	--	--	--	--
4981	375	ND	E	5/16/2012	ND	--	--	--	--
4982	376	ND	E	--	ND	--	--	--	--
4983	377	ND	E	7/10/2012	ND	--	--	--	--
5001	378	ND	F	7/9/2012	ND	--	--	--	--
5002	379	ND	F	8/3/12	ND	--	--	--	--
5003	380	ND	F	6/11/2012	ND	--	--	--	--
5021	381	ND	F	5/15/2012	ND	--	--	--	--
5022	382	ND	F	5/16/2012	ND	--	--	--	--
5023	383	ND	F	5/16/2012	ND	--	--	--	--
5024	384	ND	F	5/16/2012	ND	--	--	--	--
5041	385	ND	F	6/11/2012	ND	--	--	--	--
5042	386	ND	F	6/6/12	ND	--	--	--	--
5043	387	ND	F	8/28/12	ND	--	--	--	--
6021	388	SD	A	8/20/12	ND	--	--	--	--
6022	389	SD	A	5/14/2012	ND	--	--	--	--
6023	390	SD	A	7/6/2012	ND	--	--	--	--
6041	391	SD	A	8/21/12	ND	--	--	--	--
6061	392	SD	A	8/21/12	ND	--	--	--	--
6081	393	SD	A	8/28/12	ND	--	--	--	--
6082	394	SD	A	7/6/2012	ND	--	--	--	--
6083	395	SD	A	6/14/2012	ND	--	--	--	--
6101	396	SD	A	8/30/12	ND	--	--	--	--

Table A.5. HPLC-FD results for 2011 hard red spring wheat survey samples (continued).

6121	397	SD	B	6/12/2012	ND	--	--	--	--
6122	398	SD	B	7/6/2012	ND	--	--	--	--
6141	399	SD	B	8/21/12	ND	--	--	--	--
6142	400	SD	B	5/15/2012	ND	--	--	--	--
6161	401	SD	B	7/6/2012	ND	--	--	--	--
6162	402	SD	B	7/9/2012	ND	--	--	--	--
6163	403	SD	B	8/8/12	ND	--	--	--	--
6164	404	SD	B	5/15/2012	ND	--	--	--	--
6181	405	SD	B	5/15/2012	ND	--	--	--	--
6182	406	SD	B	8/3/12	ND	--	--	--	--
6183	407	SD	B	8/8/12	ND	--	--	--	--
6184	408	SD	B	8/20/12	ND	--	--	--	--
6201	409	SD	B	5/14/2012	ND	-	-	-	--
6202	410	SD	B	7/9/2012	ND	--	--	--	--
6221	411	SD	B	5/15/2012	ND	--	--	--	--
6222	412	SD	B	7/6/2012	ND	--	--	--	--
6223	413	SD	B	7/5/2012	ND	--	--	--	--
6224	414	SD	B	6/14/2012	ND	--	--	--	--
6241	415	SD	B	8/28/12	ND	--	--	--	--
6242	416	SD	B	7/9/2012	ND	--	--	--	--
6243	417	SD	B	7/9/2012	ND	--	--	--	--
6244	418	SD	B	6/11/2012	ND	--	--	--	--
6261	419	SD	B	5/14/2012	ND	--	--	--	--
6262	420	SD	B	5/14/2012	ND	--	--	--	--
6263	421	SD	B	8/24/2012	ND	--	--	--	--
6281	422	SD	C	8/27/2012	ND	--	--	--	--
6282	423	SD	C	8/28/12	ND	--	--	--	--
6301	424	SD	C	8/24/2012	ND	--	--	--	--
6302	425	SD	C	5/30/2012	ND	--	--	--	--
6321	426	SD	C	7/9/2012	ND	--	--	--	--
6322	427	SD	C	7/6/2012	ND	--	--	--	--
6323	428	SD	C	7/3/2012	ND	--	--	--	--
6361	429	SD	C	8/27/2012	ND	--	--	--	--
6401	430	SD	C	8/8/12	ND	--	--	--	--
6421	431	SD	C	6/14/2012	ND	--	--	--	--
6422	432	SD	C	7/6/2012	ND	--	--	--	--
6423	433	SD	C	5/16/2012	ND	--	--	--	--
6561	434	SD	B	8/29/12	ND	--	--	--	--
6621	435	SD	B	6/4/2012	ND	--	--	--	--
6622	436	SD	B	8/29/12	ND	--	--	--	--

Table A.5. HPLC-FD results for 2011 hard red spring wheat survey samples (continued).

6623	437	SD	B	8/28/12	ND	--	--	--	--
6701	438	SD	B	5/16/2012	ND	--	--	--	--
6702	439	SD	B	8/8/12	ND	--	--	--	--
6703	440	SD	B	7/3/2012	ND	--	--	--	--
6704	441	SD	B	7/3/2012	ND	--	--	--	--

[a] Retention time of peak
[b] Height of peak
[c] Result of confirmatory test
[d] Not detected
[e] Not recorded

Table A.6. HPLC-FD results for 2012 hard red spring wheat survey samples.

Original Sample #	Julie Sample #	State	Region	HPLC-FD					
				Analysis Date	Ret. Time[a] (min)	Area	Ht.[b]	OTA (ng/g)	Conf.[c]
1	128	MN	B	7/31/2012	ND[d]	--	--	--	--
2	165	MN	B	7/31/2012	ND	--	--	--	--
3	192	MN	B	8/1/2012	ND	--	--	--	--
21	415	MN	B	7/17/2012	ND	--	--	--	--
22	179	MN	B	8/2/2012	ND	--	--	--	--
23	158	MN	B	8/2/2012	ND	--	--	--	--
24	26	MN	B	8/2/2012	ND	--	--	--	--
25	163	MN	B	8/3/2012	ND	--	--	--	--
26	217	MN	B	8/2/2012	ND	--	--	--	--
61	106	MN	A	8/7/2012	ND	--	--	--	--
62	177	MN	A	8/7/2012	ND	--	--	--	--
63	147	MN	A	8/17/2012	ND	--	--	--	--
64	80	MN	A	8/17/2012	ND	--	--	--	--
65	150	MN	A	8/11/2012	ND	--	--	--	--
66	141	MN	A	8/11/2012	ND	--	--	--	--
67	224	MN	A	8/25/2012	ND	--	--	--	--
68	301	MN	A	9/5/2012	ND	--	--	--	--
69	330	MN	A	9/5/2012	ND	--	--	--	--
81	108	MN	A	--[e]	ND	--	--	--	--
101	124	MN	A	8/3/2012	ND	--	--	--	--
102	208	MN	A	8/7/2012	ND	--	--	--	--
103	201	MN	A	8/7/2012	ND	--	--	--	--
104	417	MN	A	8/8/2012	ND	--	--	--	--
105	360	MN	A	8/8/2012	ND	--	--	--	--
106	394	MN	A	8/8/2012	ND	--	--	--	--
107	411	MN	A	8/7/2012	ND	--	--	--	--
108	407	MN	A	8/14/2012	ND	--	--	--	--
109	183	MN	A	8/13/2012	ND	--	--	--	--
121	426	MN	A	7/23/2012	ND	--	--	--	--
122	357	MN	A	7/28/2012	ND	--	--	--	--
123	435	MN	A	7/23/2012	ND	--	--	--	--
124	403	MN	A	7/26/2012	ND	--	--	--	--
125	29	MN	A	7/28/2012	ND	--	--	--	--
126	164	MN	A	8/2/2012	ND	--	--	--	--
127	113	MN	A	8/1/2012	ND	--	--	--	--
141	135	MN	A	8/3/2012	ND	--	--	--	--

Table A.6. HPLC-FD results for 2012 hard red spring wheat survey samples (continued).

142	69	MN	A	7/30/2012	ND	--	--	--	--
143	110	MN	A	7/25/2012	ND	--	--	--	--
144	173	MN	A	8/2/2012	ND	--	--	--	--
145	104	MN	A	8/2/2012	ND	--	--	--	--
161	103	MN	A	8/2/2012	ND	--	--	--	--
162	207	MN	A	7/31/2012	ND	--	--	--	--
163	88	MN	A	8/7/2012	ND	--	--	--	--
164	67	MN	A	8/1/2012	ND	--	--	--	--
165	166	MN	A	7/29/2012	ND	--	--	--	--
166	117	MN	A	8/5/2012	ND	--	--	--	--
167	120	MN	A	7/26/2012	ND	--	--	--	--
168	68	MN	A	8/9/2012	ND	--	--	--	--
169	62	MN	A	8/5/2012	ND	--	--	--	--
181	175	MN	A	7/27/2012	ND	--	--	--	--
182	180	MN	A	8/3/2012	ND	--	--	--	--
183	95	MN	A	8/2/2012	ND	--	--	--	--
184	159	MN	A	7/31/2012	ND	--	--	--	--
201	383	MN	A	8/9/2012	ND	--	--	--	--
202	24	MN	A	7/30/2012	ND	--	--	--	--
203	172	MN	A	7/30/2012	ND	--	--	--	--
204	389	MN	A	8/9/2012	ND	--	--	--	--
205	98	MN	A	8/10/2012	ND	--	--	--	--
321	184	MN	A	8/10/2012	ND	--	--	--	--
501	202	MN	B	8/1/2012	ND	--	--	--	--
502	123	MN	B	8/2/2012	ND	--	--	--	--
503	174	MN	B	8/2/2012	ND	--	--	--	--
581	338	MN	B	7/23/2012	ND	--	--	--	--
601	30	MN	B	7/26/2012	ND	--	--	--	--
602	15	MN	B	7/26/2012	ND	--	--	--	--
603	16	MN	B	7/26/2012	ND	--	--	--	--
604	17	MN	B	7/26/2012	ND	--	--	--	--
605	368	MN	B	--	ND	--	--	--	--
611	SNF	MN	B	--	--	--	--	--	--
614	SNF	MN	B	--	--	--	--	--	--
2181	186	MT	A	8/17/2012	ND	--	--	--	--
2182	101	MT	A	8/17/2012	ND	--	--	--	--
2183	239	MT	A	8/28/2012	ND	--	--	--	--
2184	264	MT	A	8/28/2012	ND	--	--	--	--
2185	321	MT	A	8/28/2012	ND	--	--	--	--
2201	52	MT	A	8/25/2012	ND	--	--	--	--

Table A.6. HPLC-FD results for 2012 hard red spring wheat survey samples (continued).

2202	51	MT	A	8/23/2012	ND	--	--	--	--
2203	50	MT	A	8/21/2012	ND	--	--	--	--
2221	284	MT	A	8/24/2012	ND	--	--	--	--
2222	142	MT	A	8/29/2012	ND	--	--	--	--
2223	307	MT	A	9/10/2012	ND	--	--	--	--
2224	334	MT	A	9/10/2012	ND	--	--	--	--
2225	310	MT	A	9/10/2012	ND	--	--	--	--
2241	375	MT	A	8/11/2012	ND	--	--	--	--
2242	370	MT	A	8/8/2012	ND	--	--	--	--
2243	282	MT	A	8/15/2012	ND	--	--	--	--
2244	261	MT	A	8/16/2012	ND	--	--	--	--
2245	276	MT	A	8/16/2012	ND	--	--	--	--
2261	241	MT	A	8/20/2012	ND	--	--	--	--
2262	236	MT	A	8/23/2012	ND	--	--	--	--
2263	243	MT	A	8/21/2012	ND	--	--	--	--
2264	235	MT	A	8/21/2012	ND	--	--	--	--
2281	171	MT	A	8/2/2012	ND	--	--	--	--
2282	221	MT	A	8/14/2012	ND	--	--	--	--
2283	262	MT	A	8/21/2012	ND	--	--	--	--
2301	253	MT	A	8/20/2012	ND	--	--	--	--
2302	311	MT	A	8/20/2012	ND	--	--	--	--
2303	40	MT	A	8/29/2012	10.092	402	39	0.30	NA[f]
2304	39	MT	A	8/30/2012	ND	--	--	--	--
2305	38	MT	A	8/29/2012	ND	--	--	--	--
2321	263	MT	A	8/22/2012	ND	--	--	--	--
2322	37	MT	A	8/30/2012	ND	--	--	--	--
2323	285	MT	A	8/12/2012	ND	--	--	--	--
2341	269	MT	A	8/17/2012	ND	--	--	--	--
2342	279	MT	A	8/23/2012	ND	--	--	--	--
2343	314	MT	A	8/23/2012	ND	--	--	--	--
2344	302	MT	A	9/6/2012	ND	--	--	--	--
2345	271	MT	A	9/8/2012	ND	--	--	--	--
2361	246	MT	B	8/23/2012	ND	--	--	--	--
2362	231	MT	B	8/24/2012	ND	--	--	--	--
2363	281	MT	B	8/24/2012	ND	--	--	--	--
2364	291	MT	B	8/27/2012	ND	--	--	--	--
2365	341	MT	B	8/27/2012	ND	--	--	--	--
2381	359	MT	B	7/31/2012	ND	--	--	--	--
2382	367	MT	B	7/31/2012	ND	--	--	--	--
2383	129	MT	B	8/8/2012	ND	--	--	--	--

Table A.6. HPLC-FD results for 2012 hard red spring wheat survey samples (continued).

2384	194	MT	B	8/8/2012	ND	--	--	--	--
2385	220	MT	B	8/8/2012	ND	--	--	--	--
2401	121	MT	B	8/8/2012	ND	--	--	--	--
2421	200	MT	B	8/6/2012	ND	--	--	--	--
2422	382	MT	B	8/9/2012	ND	--	--	--	--
2423	237	MT	B	8/20/2012	ND	--	--	--	--
2424	283	MT	B	9/6/2012	ND	--	--	--	--
2441	387	MT	B	7/30/2012	ND	--	--	--	--
2442	134	MT	B	8/16/2012	ND	--	--	--	--
2443	273	MT	B	8/14/2012	ND	--	--	--	--
2444	55	MT	B	8/31/2012	ND	--	--	--	--
2445	36	MT	B	8/31/2012	ND	--	--	--	--
2446	35	MT	B	8/31/2012	ND	--	--	--	--
2461	139	MT	B	8/6/2012	ND	--	--	--	--
2462	136	MT	B	8/6/2012	ND	--	--	--	--
2463	293	MT	B	8/14/2012	ND	--	--	--	--
2464	340	MT	B	8/21/2012	ND	--	--	--	--
2465	294	MT	B	8/14/2012	ND	--	--	--	--
2466	331	MT	B	--	ND	--	--	--	--
2467	295	MT	B	8/21/2012	ND	--	--	--	--
2468	336	MT	B	8/21/2012	ND	--	--	--	--
2481	238	MT	B	8/12/2012	ND	--	--	--	--
2501	226	MT	B	8/23/2012	ND	--	--	--	--
2502	333	MT	B	8/22/2012	ND	--	--	--	--
2503	287	MT	B	8/22/2012	ND	--	--	--	--
2504	229	MT	B	8/23/2012	ND	--	--	--	--
2505	335	MT	B	8/28/2012	ND	--	--	--	--
2506	337	MT	B	8/28/2012	ND	--	--	--	--
2507	277	MT	B	8/27/2012	ND	--	--	--	--
2508	275	MT	B	8/27/2012	ND	--	--	--	--
2521	216	MT	C	8/29/2012	ND	--	--	--	--
2522	160	MT	C	8/31/2012	ND	--	--	--	--
2541	34	MT	C	8/28/2012	ND	--	--	--	--
2561	339	MT	C	8/13/2012	ND	--	--	--	--
2721	332	MT	E	8/21/2012	ND	--	--	--	--
2722	327	MT	E	8/27/2012	ND	--	--	--	--
2741	89	MT	E	8/27/2012	ND	--	--	--	--
2742	319	MT	E	9/12/2012	ND	--	--	--	--
2781	313	MT	E	9/7/2012	ND	--	--	--	--
3001	161	MT	D	9/6/2012	ND	--	--	--	--

Table A.6. HPLC-FD results for 2012 hard red spring wheat survey samples (continued).

3041	153	MT	D	8/8/2012	ND	--	--	--	--
3061	198	MT	D	8/10/2012	ND	--	--	--	--
3081	296	MT	D	8/10/2012	ND	--	--	--	--
4001	255	ND	A	8/13/2012	ND	--	--	--	--
4002	233	ND	A	8/24/2012	ND	--	--	--	--
4003	249	ND	A	8/24/2012	ND	--	--	--	--
4004	270	ND	A	8/27/2012	ND	--	--	--	--
4021	260	ND	A	8/28/2012	ND	--	--	--	--
4022	329	ND	A	8/27/2012	ND	--	--	--	--
4041	21	ND	A	8/11/2012	ND	--	--	--	--
4042	102	ND	A	8/8/2012	10.107	1907	180	1.56	+
4043	274	ND	A	8/21/2012	ND	--	--	--	--
4044	306	ND	A	8/22/2012	ND	--	--	--	--
4061	59	ND	A	8/30/2012	ND	--	--	--	--
4062	58	ND	A	8/30/2012	ND	--	--	--	--
4063	57	ND	A	8/30/2012	ND	--	--	--	--
4064	56	ND	A	8/30/2012	ND	--	--	--	--
4065	46	ND	A	8/30/2012	ND	--	--	--	--
4066	47	ND	A	8/30/2012	ND	--	--	--	--
4081	288	ND	A	8/22/2012	ND	--	--	--	--
4082	245	ND	A	8/23/2012	ND	--	--	--	--
4083	232	ND	A	8/23/2012	ND	--	--	--	--
4084	266	ND	A	8/23/2012	ND	--	--	--	--
4085	228	ND	A	8/24/2012	ND	--	--	--	--
4086	244	ND	A	8/24/2012	ND	--	--	--	--
4087	48	ND	A	8/31/2012	ND	--	--	--	--
4088	49	ND	A	8/29/2012	ND	--	--	--	--
4089	53	ND	A	8/29/2012	ND	--	--	--	--
4090	325	ND	A	8/31/2012	ND	--	--	--	--
4101	328	ND	A	8/29/2012	ND	--	--	--	--
4121	125	ND	B	8/13/2012	ND	--	--	--	--
4122	190	ND	B	8/13/2012	ND	--	--	--	--
4123	107	ND	B	8/13/2012	ND	--	--	--	--
4124	278	ND	B	8/21/2012	ND	--	--	--	--
4125	225	ND	B	8/21/2012	ND	--	--	--	--
4126	144	ND	B	8/21/2012	ND	--	--	--	--
4141	148	ND	A	8/14/2012	ND	--	--	--	--
4142	406	ND	A	8/6/2012	ND	--	--	--	--
4143	143	ND	A	8/10/2012	ND	--	--	--	--
4144	22	ND	A	8/10/2012	ND	--	--	--	--

Table A.6. HPLC-FD results for 2012 hard red spring wheat survey samples (continued).

4145	195	ND	A	8/14/2012	ND	--	--	--	--
4146	227	ND	A	8/16/2012	ND	--	--	--	--
4147	248	ND	A	8/17/2012	ND	--	--	--	--
4148	242	ND	A	8/16/2012	ND	--	--	--	--
4161	268	ND	A	8/6/2012	ND	--	--	--	--
4162	292	ND	A	8/2/2012	ND	--	--	--	--
4163	308	ND	A	8/2/2012	ND	--	--	--	--
4164	309	ND	A	8/17/2012	ND	--	--	--	--
4165	256	ND	A	8/10/2012	ND	--	--	--	--
4181	320	ND	B	7/31/2012	ND	--	--	--	--
4182	324	ND	B	8/1/2012	ND	--	--	--	--
4183	252	ND	B	8/3/2012	ND	--	--	--	--
4184	257	ND	B	8/16/2012	ND	--	--	--	--
4185	130	ND	B	8/19/2012	ND	--	--	--	--
4201	222	ND	B	8/12/2012	ND	--	--	--	--
4202	247	ND	B	8/27/2012	ND	--	--	--	--
4203	322	ND	B	8/27/2012	ND	--	--	--	--
4221	93	ND	B	8/7/2012	ND	--	--	--	--
4223	316	ND	B	8/17/2012	ND	--	--	--	--
4224	300	ND	B	8/17/2012	ND	--	--	--	--
4225	289	ND	B	8/17/2012	ND	--	--	--	--
4226	223	ND	B	8/24/2012	ND	--	--	--	--
4227	286	ND	B	8/24/2012	ND	--	--	--	--
4228	240	ND	B	8/24/2012	ND	--	--	--	--
4229	265	ND	B	8/24/2012	ND	--	--	--	--
4241	414	ND	C	7/31/2012	ND	--	--	--	--
4242	416	ND	C	7/30/2012	ND	--	--	--	--
4243	436	ND	C	7/30/2012	ND	--	--	--	--
4244	64	ND	C	8/1/2012	ND	--	--	--	--
4245	82	ND	C	7/31/2012	ND	--	--	--	--
4246	85	ND	C	7/31/2012	ND	--	--	--	--
4247	290	ND	C	8/17/2012	ND	--	--	--	--
4248	251	ND	C	8/13/2012	ND	--	--	--	--
4249	272	ND	C	8/13/2012	ND	--	--	--	--
4261	347	ND	B	8/1/2012	ND	--	--	--	--
4262	373	ND	B	7/29/2012	ND	--	--	--	--
4263	353	ND	B	8/1/2012	ND	--	--	--	--
4264	410	ND	B	8/1/2012	ND	--	--	--	--
4281	97	ND	C	7/30/2012	ND	--	--	--	--
4282	76	ND	C	7/30/2012	ND	--	--	--	--

Table A.6. HPLC-FD results for 2012 hard red spring wheat survey samples (continued).

4283	77	ND	C	7/30/2012	ND	--	--	--	--
4284	109	ND	C	8/2/2012	ND	--	--	--	--
4285	87	ND	C	7/30/2012	ND	--	--	--	--
4286	131	ND	C	8/2/2012	ND	--	--	--	--
4287	138	ND	C	8/2/2012	ND	--	--	--	--
4288	100	ND	C	8/2/2012	ND	--	--	--	--
4289	178	ND	C	8/8/2012	ND	--	--	--	--
4301	390	ND	B	8/2/2012	ND	--	--	--	--
4302	187	ND	B	8/14/2012	ND	--	--	--	--
4303	381	ND	B	8/13/2012	ND	--	--	--	--
4304	188	ND	B	8/14/2012	ND	--	--	--	--
4305	189	ND	B	8/14/2012	ND	--	--	--	--
4321	215	ND	B	8/10/2012	ND	--	--	--	--
4322	185	ND	B	8/10/2012	ND	--	--	--	--
4323	280	ND	B	8/18/2012	ND	--	--	--	--
4324	298	ND	B	8/17/2012	ND	--	--	--	--
4325	315	ND	B	8/17/2012	ND	--	--	--	--
4326	145	ND	B	9/4/2012	ND	--	--	--	--
4327	140	ND	B	8/18/2012	ND	--	--	--	--
4328	152	ND	B	8/21/2012	ND	--	--	--	--
4341	86	ND	C	8/1/2012	ND	--	--	--	--
4342	116	ND	C	7/30/2012	ND	--	--	--	--
4343	94	ND	C	8/1/2012	ND	--	--	--	--
4344	380	ND	C	8/8/2012	ND	--	--	--	--
4345	376	ND	C	8/9/2012	ND	--	--	--	--
4346	25	ND	C	8/10/2012	ND	--	--	--	--
4347	114	ND	C	8/13/2012	ND	--	--	--	--
4348	146	ND	C	8/13/2012	ND	--	--	--	--
4349	105	ND	C	8/13/2012	ND	--	--	--	--
4361	379	ND	D	8/6/2012	ND	--	--	--	--
4362	374	ND	D	8/6/2012	ND	--	--	--	--
4363	73	ND	D	8/10/2012	ND	--	--	--	--
4364	96	ND	D	8/10/2012	ND	--	--	--	--
4365	214	ND	D	8/10/2012	ND	--	--	--	--
4381	193	ND	A	8/6/2012	ND	--	--	--	--
4382	197	ND	A	8/6/2012	ND	--	--	--	--
4383	203	ND	A	--	ND	--	--	--	--
4401	364	ND	A	7/29/2012	ND	--	--	--	--
4402	420	ND	A	--	ND	--	--	--	--
4403	355	ND	A	7/23/2012	ND	--	--	--	--

Table A.6. HPLC-FD results for 2012 hard red spring wheat survey samples (continued).

4404	162	ND	A	8/3/2012	ND	--	--	--	--
4405	182	ND	A	7/31/2012	ND	--	--	--	--
4406	112	ND	A	8/6/2012	ND	--	--	--	--
4407	122	ND	A	8/6/2012	ND	--	--	--	--
4408	132	ND	A	8/8/2012	ND	--	--	--	--
4409	318	ND	A	8/17/2012	ND	--	--	--	--
4421	399	ND	D	8/1/2012	ND	--	--	--	--
4422	388	ND	D	8/1/2012	ND	--	--	--	--
4441	349	ND	D	7/30/2012	ND	--	--	--	--
4442	90	ND	D	8/10/2012	ND	--	--	--	--
4461	405	ND	B	8/3/2012	ND	--	--	--	--
4462	385	ND	B	8/3/2012	ND	--	--	--	--
4481	365	ND	B	7/30/2012	ND	--	--	--	--
4482	305	ND	B	8/17/2012	ND	--	--	--	--
4483	323	ND	B	8/17/2012	ND	--	--	--	--
4501	83	ND	E	7/24/2012	ND	--	--	--	--
4502	61	ND	E	8/7/2012	ND	--	--	--	--
4521	155	ND	A	8/2/2012	10.184	650	62	0.60	NA
4522	133	ND	A	8/10/2012	ND	--	--	--	--
4523	191	ND	A	8/10/2012	ND	--	--	--	--
4541	402	ND	E	7/27/2012	ND	--	--	--	--
4542	398	ND	E	7/26/2012	ND	--	--	--	--
4543	60	ND	E	7/30/2012	ND	--	--	--	--
4544	118	ND	E	7/31/2012	ND	--	--	--	--
4545	119	ND	E	7/30/2012	ND	--	--	--	--
4561	378	ND	B	7/30/2012	ND	--	--	--	--
4562	12	ND	B	8/10/2012	ND	--	--	--	--
4563	149	ND	B	8/10/2012	ND	--	--	--	--
4564	430	ND	B	8/10/2012	ND	--	--	--	--
4565	170	ND	B	8/10/2012	ND	--	--	--	--
4566	267	ND	B	8/17/2012	ND	--	--	--	--
4567	297	ND	B	8/17/2012	ND	--	--	--	--
4568	326	ND	B	8/11/2012	ND	--	--	--	--
4581	10	ND	F	7/27/2012	10.055	287	29	0.21	NA
4582	27	ND	F	7/27/2012	ND	--	--	--	--
4583	65	ND	F	8/2/2012	ND	--	--	--	--
4584	66	ND	F	8/2/2012	ND	--	--	--	--
4585	181	ND	F	8/1/2012	ND	--	--	--	--
4601	28	ND	F	7/23/2012	ND	--	--	--	--
4602	424	ND	F	7/24/2012	ND	--	--	--	--

Table A.6. HPLC-FD results for 2012 hard red spring wheat survey samples (continued).

4603	348	ND	F	7/27/2012	ND	--	--	--	--
4604	354	ND	F	7/24/2012	ND	--	--	--	--
4605	18	ND	F	7/24/2012	ND	--	--	--	--
4621	74	ND	B	7/30/2012	ND	--	--	--	--
4622	75	ND	B	8/2/2012	ND	--	--	--	--
4623	206	ND	B	8/2/2012	ND	--	--	--	--
4641	78	ND	C	7/30/2012	ND	--	--	--	--
4642	199	ND	C	8/10/2012	ND	--	--	--	--
4643	209	ND	C	8/2/2012	ND	--	--	--	--
4644	72	ND	C	8/2/2012	ND	--	--	--	--
4661	14	ND	C	7/23/2012	ND	--	--	--	--
4662	7	ND	C	7/24/2012	ND	--	--	--	--
4663	11	ND	C	7/24/2012	ND	--	--	--	--
4664	19	ND	C	7/28/2012	ND	--	--	--	--
4681	219	ND	D	7/31/2012	ND	--	--	--	--
4682	126	ND	D	8/1/2012	ND	--	--	--	--
4683	91	ND	D	8/5/2012	ND	--	--	--	--
4684	92	ND	D	8/7/2012	ND	--	--	--	--
4701	250	ND	D	8/17/2012	ND	--	--	--	--
4721	413	ND	D	7/31/2012	ND	--	--	--	--
4722	81	ND	D	8/7/2012	ND	--	--	--	--
4722	205	ND	D	8/7/2012	ND	--	--	--	--
4741	304	ND	D	8/17/2012	ND	--	--	--	--
4761	395	ND	D	7/31/2012	ND	--	--	--	--
4762	397	ND	D	7/31/2012	ND	--	--	--	--
4763	157	ND	D	8/2/2012	ND	--	--	--	--
4764	204	ND	D	8/2/2012	ND	--	--	--	--
4765	156	ND	D	8/6/2012	ND	--	--	--	--
4766	211	ND	D	8/7/2012	ND	--	--	--	--
4767	167	ND	D	8/6/2012	ND	--	--	--	--
4768	408	ND	D	8/6/2012	ND	--	--	--	--
4781	352	ND	D	7/29/2012	ND	--	--	--	--
4782	401	ND	D	7/29/2012	ND	--	--	--	--
4783	412	ND	D	7/31/2012	ND	--	--	--	--
4801	351	ND	D	8/1/2012	ND	--	--	--	--
4802	386	ND	D	--	ND	--	--	--	--
4803	169	ND	D	8/8/2012	10.184	2140	198	1.97	+
4804	99	ND	D	8/8/2012	ND	--	--	--	--
4805	151	ND	D	8/8/2012	10.214	594	57	0.49	NA
4806	210	ND	D	8/10/2012	ND	--	--	--	--

Table A.6. HPLC-FD results for 2012 hard red spring wheat survey samples (continued).

4807	317	ND	D	8/22/2012	ND	--	--	--	--
4808	259	ND	D	8/25/2012	ND	--	--	--	--
4821	361	ND	E	7/28/2012	ND	--	--	--	--
4822	345	ND	E	7/25/2012	ND	--	--	--	--
4823	84	ND	E	8/1/2012	ND	--	--	--	--
4824	176	ND	E	8/8/2012	ND	--	--	--	--
4841	33	ND	E	7/24/2012	ND	--	--	--	--
4842	437	ND	E	7/27/2012	ND	--	--	--	--
4843	71	ND	E	8/1/2012	ND	--	--	--	--
4844	409	ND	E	8/9/2012	ND	--	--	--	--
4845	23	ND	E	8/10/2012	ND	--	--	--	--
4861	213	ND	D	8/8/2012	ND	--	--	--	--
4862	212	ND	D	8/8/2012	ND	--	--	--	--
4863	258	ND	D	8/18/2012	ND	--	--	--	--
4881	154	ND	D	8/8/2012	ND	--	--	--	--
4882	168	ND	D	8/8/2012	ND	--	--	--	--
4883	312	ND	D	8/18/2012	ND	--	--	--	--
4884	303	ND	D	8/18/2012	ND	--	--	--	--
4885	299	ND	D	8/18/2012	ND	--	--	--	--
4886	254	ND	D	8/18/2012	ND	--	--	--	--
4901	79	ND	D	8/8/2012	ND	--	--	--	--
4921	31	ND	E	7/28/2012	ND	--	--	--	--
4941	421	ND	E	7/26/2012	ND	--	--	--	--
4942	377	ND	E	7/31/2012	ND	--	--	--	--
4961	234	ND	E	8/15/2012	ND	--	--	--	--
4962	230	ND	E	8/16/2012	ND	--	--	--	--
4981	384	ND	E	8/2/2012	ND	--	--	--	--
4982	393	ND	E	7/31/2012	ND	--	--	--	--
5001	343	ND	F	7/27/2012	ND	--	--	--	--
5002	137	ND	F	8/1/2012	ND	--	--	--	--
5021	371	ND	F	7/16/2012	ND	--	--	--	--
5022	362	ND	F	7/20/2012	ND	--	--	--	--
5023	127	ND	F	8/6/2012	ND	--	--	--	--
5041	70	ND	F	8/2/2012	ND	--	--	--	--
5042	63	ND	F	--	ND	--	--	--	--
6021	418	SD	A	7/25/2012	ND	--	--	--	--
6022	428	SD	A	7/27/2012	ND	--	--	--	--
6023	404	SD	A	8/1/2012	ND	--	--	--	--
6041	392	SD	A	7/19/2012	ND	--	--	--	--
6061	20	SD	A	8/6/2012	ND	--	--	--	--

Table A.6. HPLC-FD results for 2012 hard red spring wheat survey samples (continued).

6081	369	SD	A	7/26/2012	ND	--	--	--	--
6082	429	SD	A	8/6/2012	ND	--	--	--	--
6101	419	SD	A	7/19/2012	ND	--	--	--	--
6121	4	SD	B	7/19/2012	ND	--	--	--	--
6141	358	SD	B	7/31/2012	ND	--	--	--	--
6142	344	SD	B	8/2/2012	ND	--	--	--	--
6161	32	SD	B	7/23/2012	ND	--	--	--	--
6162	5	SD	B	7/24/2012	ND	--	--	--	--
6163	6	SD	B	7/24/2012	ND	--	--	--	--
6181	427	SD	B	7/20/2012	ND	--	--	--	--
6182	350	SD	B	7/19/2012	ND	--	--	--	--
6183	366	SD	B	7/20/2012	ND	--	--	--	--
6201	1	SD	B	7/24/2012	ND	--	--	--	--
6202	8	SD	B	7/24/2012	ND	--	--	--	--
6221	425	SD	B	7/19/2012	ND	--	--	--	--
6222	54	SD	B	7/19/2012	ND	--	--	--	--
6223	45	SD	B	7/19/2012	ND	--	--	--	--
6224	44	SD	B	7/19/2012	ND	--	--	--	--
6241	431	SD	B	7/19/2012	ND	--	--	--	--
6242	422	SD	B	7/27/2012	ND	--	--	--	--
6261	391	SD	B	7/14/2012	ND	--	--	--	--
6262	346	SD	B	7/24/2012	ND	--	--	--	--
6263	423	SD	B	7/30/2012	ND	--	--	--	--
6281	363	SD	C	7/24/2012	ND	--	--	--	--
6301	115	SD	C	8/3/2012	ND	--	--	--	--
6302	396	SD	C	7/24/2012	ND	--	--	--	--
6321	400	SD	C	7/30/2012	ND	--	--	--	--
6322	372	SD	C	7/23/2012	ND	--	--	--	--
6323	356	SD	C	--	ND	--	--	--	--
6361	432	SD	C	7/26/2012	ND	--	--	--	--
6362	433	SD	C	8/2/2012	ND	--	--	--	--
6401	13	SD	C	7/23/2012	ND	--	--	--	--
6421	2	SD	C	7/25/2012	ND	--	--	--	--
6422	3	SD	C	--	ND	--	--	--	--
6561	43	SD	B	7/23/2012	ND	--	--	--	--
6621	218	SD	B	7/20/2012	ND	--	--	--	--
6622	111	SD	B	8/5/2012	ND	--	--	--	--
6623	196	SD	B	7/28/2012	ND	--	--	--	--
6701	42	SD	B	7/13/2012	ND	--	--	--	--
6702	434	SD	B	7/19/2012	ND	--	--	--	--

Table A.6. HPLC-FD results for 2012 hard red spring wheat survey samples (continued).

6703	9	SD	B	7/19/2012	ND	--	--	--	--
6704	41	SD	B	7/20/2012	ND	--	--	--	--
6705	342	SD	B	7/30/2012	ND	--	--	--	--

[a] Retention time of peak

[b] Height of peak

[c] Result of confirmatory test

[d] Not detected

[e] Not recorded

[f] Not applicable as only samples with >1 ng/g were subjected to confirmatory testing