QUANTITATIVE ANALYSIS FOR MEASURING LOWER LEVELS OF *FUSARIUM* MYCOTOXINS IN WHEAT AND BARLEY USING HIGH-RESOLUTION MASS SPECTROMETRY

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Pradeep Samadhan Sarkate

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

MASTER OF SCIENCE

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ABSTRACT

This project involved the application of Quadrupole Time of Flight (QTOF) technology in quantitating the low concentrations of multiple *Fusarium* mycotoxins in barley and wheat, also focused on simplified sample extraction protocols such as 'dilute and shoot.' Ground samples of wheat and barley were extracted with acetonitrile-water-acetic acid solution (70:29:1 v/v/v). The quantitation was performed using a post spiking matrix-matched calibration curve approach. The method was linear over the range of 1.56 – 100 μg/kg for the toxins deoxynivalenol (DON), deoxynivalenol-3-glucoside (D3G), 3-acetyl deoxynivalenol (3-ADON), 15-acetyl deoxynivalenol (15-ADON), diacetoxyscirpenol (DAS), fusarenon-X (FUS-X), nivalenol (NIV), Neosolaniol (NEO), T2, and HT2 toxin. Zearalenone (ZEA). The recovery of the 11 mycotoxins in wheat and barley matrices at two levels were within 60 - 130.1%, and the relative standard deviation (RSD) of the replicate sample assay fell within 5 to 40%. Overall, this method was successfully validated for all the *Fusarium* toxins.

ACKNOWLEDGMENTS

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1. INTRODUCTION

Fusarium head blight (FHB), or scab, is a serious fungal disease that impacts a number of cereal crops like barley, wheat, and rye in many of the world's major production areas. FHB infects the head or inflorescence of cereal and can reduce kernel number and size, which in turn has a major impact on grain yield. Grain quality can also be impacted. However, the greatest concern is the production of a number of mycotoxins associated with the *Fusarium* pathogens. Mycotoxins can be phytotoxic and also pose a risk to human and animal health (Gruber-Dorninger et al., 2019). Globally, about 25% of food crops are affected by mycotoxins, leading to billions of dollars losses annually. Many countries have limits on levels of mycotoxins present in food and feed grains (Eskola et al., 2019).

Mycotoxins are secondary fungal metabolites which are toxic to human, animals and plants. Trichothecenes are the major class of mycotoxins associated with FHB and include deoxynivalenol (DON), deoxynivalenol-3-glucoside (D3G), 3-acetyl deoxynivalenol (3-ADON), 15-acetyl deoxynivalenol (15-ADON), diacetoxyscirpenol (DAS), fusarenon-X (FUS-X), nivalenol (NIV), Neosolaniol(NEO), T2, and HT2 toxin. Zearalenone (ZEA) is also sometimes associated with FHB (Berthiller et al., 2013). The toxins occurring on FHB infected grain are dependent upon the species and chemotype of *Fusarium* pathogen, environmental conditions, and geographic location. *Fusarium graminearum* and DON are the most common pathogen and toxin associated with small grains in North America. However, grain can be infected by several species of *Fusarium* and can be contaminated with two or more mycotoxins. In addition to the trichothecenes and ZEA, FHB infected grain may also contain, one of several of the so-called emerging mycotoxins. These include fusaproliferin, beauvericin, enniatins, and moniliformin.

Although not commonly associated with FHB, *Fusarium* infection of grain can also result in contamination with fumonisins (Jestoi et al., 2008).

Given the food safety and economic concerns, widespread occurrence, and regulations regarding mycotoxins, testing is extremely important. Traditional strategies for the determination of mycotoxins incorporate enzyme-linked immunosorbent assays (ELISA), thin-layer chromatography (TLC), high pressure liquid chromatography (HPLC) and gas chromatography (GC). The grain trade tends to rely on rapid methods, that are often in the form of test kits. Test kits are often based on ELISA, but other platforms include, membrane-based immunoassays, fluorescence polarization immunoassays and fluorometric assays (Nolan et al., 2019). Research and analytical testing laboratories, on the other hand, tend to utilize chromatographic methods, which are often coupled with some form of mass spectrometry (MS). A number of factors influence the selection of instrumentation and methodology and can include lower limits of detection and quantitation (LOD and LOQ), detection of multiple toxins, required amount of sample, and speed of analysis. Price can also be a consideration with LC-MS/MS instrumentation generally costing far more than GC-MS.

Mycotoxins can be determined with GC-MS with the reasonable sensitivity, but quantitation at ppb (parts per billion) levels (µg/kg) sensitivity is not always possible for the following reasons (Kekkonen and Jestoi, 2009). First, GC-MS requires that analytes are volatile, or can be derivatized to form volatile compounds. Some mycotoxins, such as the conjugated toxin DON-3-glucoside, cannot be made volatile. In addition, when the target is multiple toxins, the same derivatizing agent may not be effective for all analytes.

As alternative to GC-MS methods, liquid chromatography with tandem mass spectrometry (LC-MS/MS) can be employed for determination of mycotoxins with the improved

selectivity and sensitivity (Kekkonen and Jestoi, 2009). LC-MS enables rapid identification of the analytes with high accuracy and reliability. Furthermore, it provides the high selectivity and simultaneous measurement of compounds at relatively low concentrations. Mycotoxins are polar in nature, easily soluble in LCMS solvents, and widely ionized in electrospray ionization (ESI) techniques. The need for LC-MS methods has really increased due to the diversity of analytes being analyzed. LC-MS/MS methods based on a triple quadrupole platform with the multiple-reaction monitoring (MRM) mode are the gold standard for the quantitation. Molecules in the sample are ionized, and the first mass spectrometer separates these by their mass-to-charge ratio (m/z). Ions of a specific m/z-ratio can then be selected and split into smaller fragments. These fragments are then introduced into the second mass spectrometer which in turn separates the fragments by their m/z-ratio and detects them. It is highly selective method since, filtering the ions at both the precursor and fragment levels reduces the background noise and improves the signal of the analyte. This is the primary reason that LC-MS/MS-based methods achieve the lowest detection limit. With the MRM method hundreds of analytes can be analyzed in a single run.

High pressure liquid chromatography with quadruple time of flight (HPLC-QTOF) is another LC-MS platform. However, it is most commonly utilized for the identification of unknown compounds based on prediction of chemical formula from accurate ion mass measurement and isotopic pattern (Romera et al., 2019). In most cases, the LOQs from the TOF instrument are slightly poorer than those from triple quad instruments as the TOF analysis is a full scan mode for the defined mass range and can resolve two close m/z values, with a mass difference of 0.001. However, there are no reports on the use of the QTOF platform for the quantitation of low levels of *Fusarium* toxins.

At the initiation of the current research, only LC-QTOF-MS was available in the Department of Plant Sciences at NDSU. The overall objective of the project was to develop an accurate quantitative multi-toxin method utilizing the Agilent 6540 UHD LC-QTOF instrument. Goals were the rapid measurement of low levels of *Fusarium* toxins such as DON, D3G, 15-ADON, NIV, NEO, DAS, FUS-X, HT-2, T-2, and ZEN using the TOF-MS full scan, and optimization of simple and easy sample extraction protocol. Chromatographic separation of 3-ADON and 15-ADON was a secondary goal. In this study, a highly sensitive method was developed for the QTOF instrument, which utilized minimum sample treatment, as opposed to solid-phase extraction columns. Minimal sample treatment simplifies analysis and minimizes solvent and reagent consumption.

2. LITERATURE REVIEW

2.1. Fusarium Head Blight (FHB)

Fusarium Head Blight (FHB), also known as *Scab*, is a disease that impacts small grains, including wheat, barley, rye, and triticale (McMullen et al., 2012). It is caused by several species in the filamentous fungal genera, *Fusarium*, and, as the name implies, it infects kernels in the spike or head of the plant. FHB occurs in many of the world's cereal production regions and is considered as one of the most devastating plant diseases. The United States Department of Agriculture (USDA) has stated that FHB is the worst plant disease to impact US cereal production since the 1950s.

The disease has the potential to reduce grain yield, kernel size, and the processing quality/food safety of the grain. Impacts on food safety are due to the production of mycotoxins by *Fusarium* and resultant contamination of the grain. As these fungal toxins can affect human and animal health, many countries have implemented regulatory guidelines for permittable levels of *Fusarium* mycotoxins in food and feed grains.

2.1.1. Pathogens and Symptoms of FHB

In North America, FHB is primarily caused by Fusarium graminearum (Friskop et al., 2018). However, specific species can vary by geographic location, and plants are sometimes also infected with multiple species, which can include F. pseudograminearum, F. avenaceum, F. poae, F. culmorum, as well as others. The source of inoculum is generally residues from small grains crops or maize. Fungal spores are wind-blown, or rain splashed from residues onto developing spikelets. Infection of the cereal head is augmented by moist and warm conditions during the course of flowering and grain development. Symptoms can manifest over the full head, or just on a few spikelets. In wheat, symptoms commonly appear as bleached spikelets or pink-orange

discoloration. When infection occurs early, kernels can be shriveled, lightweight, and dull grayish or pinkish. These are referred to as tombstone kernels.

On the other hand, in barley, symptoms often take the form of tan or dark coloring on the specific spikelets impacted. If a humid environment persists, white, pink, or orange masses characterized by spores could form along the foundation of the glumes. Reduction in kernel size is not as common in barley as for wheat heads.

2.1.2. Effects of FBH on Grain Quality

Wheat and barley are important food and feed crops globally, and FHB of these crops has led to billion-dollar losses (McMullen et al., 2012). This is through reduced yields and quality, and the consequent reductions in market pricing. The occurrence of FHB has also made other crops more attractive to growers in some areas, thus, threatening overall acreage of small grains in these regions.

FHB can impact grain yield through reduction in kernel number, caused by spikelet death, or through reduction in kernel weight. Impacts on yield are related to timing and severity of infection. While infection at later stages of kernel development may not result any significant yield reduction, early infection can, in some cases, result in the yield being lowered by as much as 80 percent. This is related to the fact that many *Fusarium* toxins show phytotoxicity, and impacts are likely larger during flowering or early in grain development. However, more significant losses are probably associated with lower quality/safety of the harvested grain, which results in the grade of the grain being reduced (Magliano and Chulze, 2013). Some quality reductions are associated with changes in chemical composition, which in turn influence processing quality. An example is the degradation of cereal proteins by *Fusarium* proteases that can occur during the colonization of the grain (Sarlin et al., 2005). This change in protein profile

can impact both malting and baking functionality. Quality reductions can also be associated with the presence of fungal metabolites. Best known is the contamination with mycotoxins, which cause food and feed safety concerns. However, with malting barley, hydrophobins produced by *Fusarium* can be a cause of beer gushing and is also of concern.

2.2. *Fusarium* Mycotoxins

Mycotoxins are perhaps the largest food safety concern with cereal grains and are also a major issue for food security. They can accumulate in infected small grains and, when consumed by humans or livestock, lead to the development of diseases. As previously stated, major mycotoxigenic fungi impacting wheat and barley belong to the filamentous genera *Fusarium*. *Fusarium* species can produce several significant classes of mycotoxins which include, the trichothecenes, zearalenone, and fumonisins. In addition, *Fusarium* species have been relatively recently reported to produce beauvericin, enniatins, fusaproliferin, fusaric acids, fusarins, and moniliform. These are often referred to as “minor” or “emerging” mycotoxins.

2.2.1. Trichothecenes

Trichothecenes are the largest group of mycotoxins, consisting of more than 150 chemically related compounds. According to their chemical structures, they are sesquiterpene compounds. Trichothecenes can be segmented into four categories: A, B, C, and D. Types A-C are based on substitution at the C-8 position of the basic carbon skeleton. The type A category includes T-2 and HT-2 toxins, as well as diacetoxyscirpenol, while the type B category includes deoxynivalenol (DON) and nivalenol (NIV). Trichothecenes are minimal in size and are amphipathic molecules having both hydrophobic and hydrophilic portions. In mammals, contact with these toxins could lead to immunological challenges, skin dermatitis, nausea, gastroenteritis, hemorrhagic lesions, and acute disease (Desjardins and Proctor, 2007). The

actions of trichothecenes on eukaryotic cells involve multiple inhibitory mechanisms, including protein, DNA, and RNA synthesis. In wheat, trichothecenes are phytotoxic, where they cause chlorosis and dwarfism.

2.2.2. Trichothecene Biosynthesis

Understanding the biosynthesis of trichothecenes is important in understanding their structural differences. This biosynthetic pathway was reviewed by McCormick et al., 2011, and only a short overview is presented here. Trichothecene biosynthesis begins with the cyclization of the terpenoid, farnesyl pyrophosphate, to form trichodiene (Figure 1).

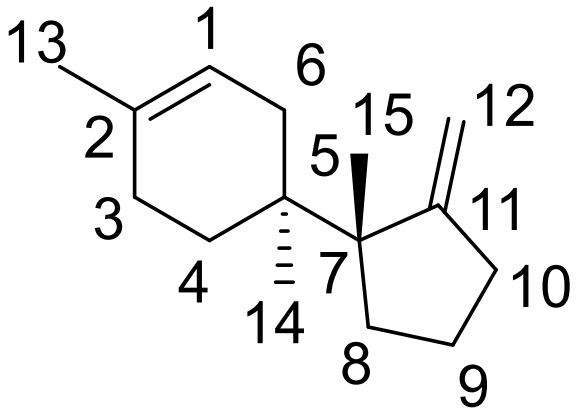

Figure 1. Basic chemical structure of trichodiene with carbon numbering.
(Adapted from https://chem.nlm.nih.gov/chemidplus/name/trichodiene).

This step is catalyzed by terpene cyclase trichodiene synthase (Tri5) and is coded for by the (*TRI5*) gene. Trichodiene is then transformed through a series of enzymatic and non-enzymatic steps. Other enzymes and the corresponding genes in the pathway follow a similar naming pattern (e.g., *tri and TRI)*. Trichodiene first undergoes a sequence of oxygenations (catalyzed by a cytochrome P450 monooxygenase, which is coded by *TRI4). TRI4 controls the* oxygenations at C-2, C-3, C-11, as well as the C-12, C-13-epoxide to form isotrichotriol. Isotrichotriol undergoes non-enzymatic isomerization and cyclization to form isotrichodermol (Figure 2), which shows the basic carbon skeleton present in all trichothecenes. Increasingly

multifaceted trichothecenes are then formed from isotrichodermol through a series of hydroxylation, acetylation, and acylation steps. There are several branches in the pathway, with the first leading to the type B compounds, DON, 3-acetyl deoxynivalenol (3-ADON), and 15-acetyl deoxynivalenol (15-ADON). These compounds have no substitution at C-4 (Figures 4, 5, and 6). Additional steps lead to hydroxylation at C-4 and formation of the type B nivalenol (NIV) (Figure 7). A third branch leads to formation of the type A trichothecenes, such as T-2 toxin (Figure 8).

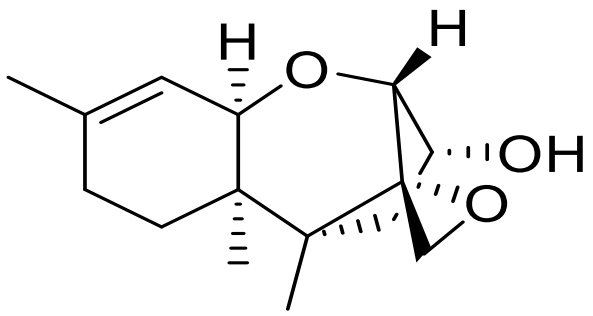

Figure 2. Basic chemical structure of isotrichodermol.
(Adopted from https://chem.nlm.nih.gov/chemidplus/name/isotrichodermol).

2.2.3. Toxicological Mechanisms of Trichothecenes

Trichothecenes have been reported to cause apoptosis and/or necrosis in the lymphoid, hematopoietic, and gastrointestinal systems resulting in leukopenia, vomiting, and diarrhea (McCormick et al. 2011). In addition, trichothecenes are toxic to the skin and testes. The toxicity of trichothecenes was covered as part of a recent review of trichothecenes in cereal grains by Foroud and coworkers (2019). They reported that the main target of toxicity is the ribosome, where trichothecenes bind and interfere with protein synthesis. The specific action is at the peptidyl transferase center, where trichothecenes disrupt peptide bond formation during translation. The 12, 13 epoxide group has long been known to be essential for toxicity. However, while epoxides are highly reactive, there appears to not be direct interaction of the epoxide with

the ribosome. It was recently proposed that epoxide is essential for stabilization of structure and aids in the interaction of the trichothecene with a binding pocket of the peptidyl transferase center of the ribosome.

The toxicity of trichothecenes varies widely and also varies between affected organisms. Differences in structure relate to substitutions at various positions of the basic carbon skeleton. For example, acetylation at C-3 has been thought to reduce toxicity in plants. While substitution at specific sites of the toxin may directly affect protein synthesis, differences in toxicity may also just be a result of uptake by the cell.

2.2.4. Type B Tricothecenes

Type B trichothecenes (Figure 3) have a keto (carbonyl) function at C-8 (e.g., nivalenol, deoxynivalenol, and trichothecin). In *Fusarium*, Type B trichothecenes typically have a C-7 hydroxyl group, but this structural feature is not present in other genera (McCormick et al., 2011). Trichothecenes produced by *Trichoderma*, *Trichothecium*, *Myrothecium*, or *Stachybotrys* lack an oxygen function at the C-3 position. The specific toxins produced by an organism are both species and strain-dependent (Foroud et al., 2019). The term chemotype is used to define the toxin profile of specific fungal strains.

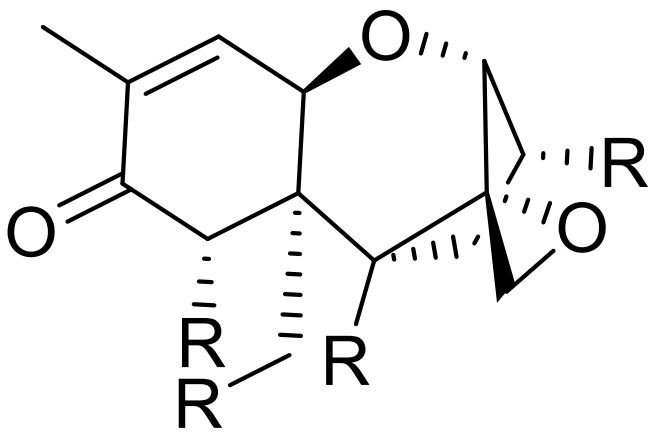

Figure 3. Basic chemical structure of type B trichothecene mycotoxins.
(adapted from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2662451/).

2.2.4.1. Deoxynivalenol, 3- and 15 Acetyl- Deoxynivalenol

Deoxynivalenol (DON) (Figure 4) is the most common mycotoxin associated with FHB in North America, as well as in other parts of the world (Friskop et al., 2018). Its production is commonly associated *with F. graminearum,* but it is also produced by other species including, *F. culmorum* (Munkvold et al., 2017). Numerous surveys of DON in cereal grains have been conducted around the world, and levels have been reported to range from <10 to more than 2,500 µg/kg (Jin and Schwarz, 2020). Incidences of contamination as high as 100% of the crop have been reported in some regional surveys. Because of its common occurrence, many countries have regulatory limits on levels of DON that can be utilized for food and feed applications. The U.S. Food and Drug Administration (FDA) has implemented advisory levels of 1 ppm (mg/Kg) DON on finished wheat products (e.g., flour, bran, and germ) that may potentially be consumed by humans.

3- and 15 acetyl- deoxynivalenol are formed by the same organisms as DON and, in terms of biosynthesis, both are immediate precursors of DON (McCormick et al., 2011). However, within *Fusarium* species, some chemotypes chemotypes that produce largely one or the other of the acetylated forms (e.g., 3- ADON and 15-ADON chemotypes) (Foroud et al., 2019). The chemotypes show not only differences in toxin profile, but also fitness and pathogenicity. The amounts of 3-ADON and 15-ADON present in contaminated grain are generally much lower than that of DON. However, there is interest in measuring these compounds, as the acetylated derivatives may display enhanced or reduced toxicity when compared to DON (Foldbjerg et al., 2012).

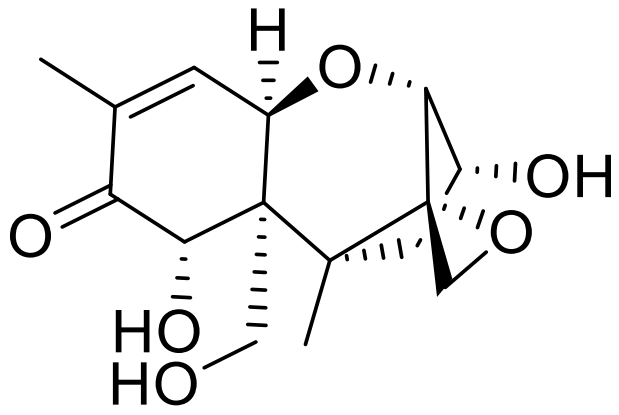

Figure 4. Chemical structure of deoxynivalenol (DON).
(Adapted from https://chem.nlm.nih.gov/chemidplus/rn/51481-10-8).

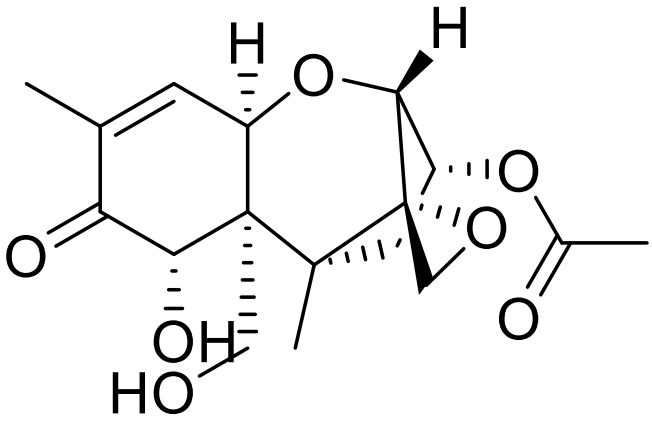

Figure 5. Chemical structure of 3-acetyl deoxynivalenol (3-ADON).
(Adapted from https://chem.nlm.nih.gov/chemidplus/rn/50722-38-8).

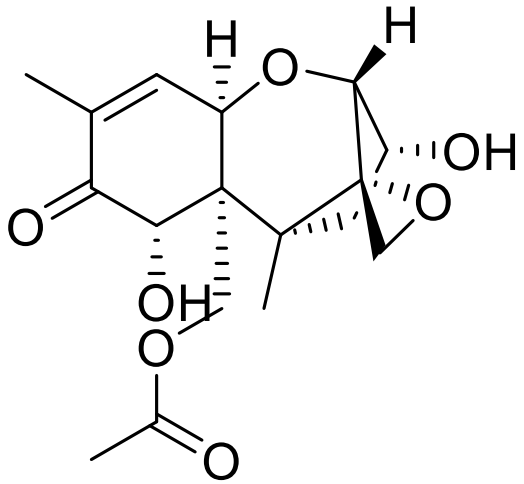

Figure 6. Chemical structure of 15-acetyl deoxynivalenol (15-ADON).
(Adapted from https://chem.nlm.nih.gov/chemidplus/rn/88337-96-6).

2.2.4.2. Nivalenol

Nivalenol (NIV), as is DON, is derived from 3, 15-acetyl-deoxynivalenol. Its formation represents a second branch of the trichothecene biosynthetic pathway (McCormick et al., 2011). Unlike DON, it is hydroxylated at the C-4 position (Figure 7). NIV has been reported to be produced by strains of *F. cerealis*, *F. poae*, *F. nivale*, *F. culmorum* as well as by *F. graminearum*. Strains of *F. gramiearum* that produce NIV are referred to as NIV chemotypes, and are of interest as NIV has greater toxicity when compared to DON (Schwarz, 2017). NIV is not commonly identified in European countries, but its occurrence has been described in the USA and some Asian countries (Berthiller et al., 2009). In the USA, NIV is generally detected at a much lower frequency than DON.

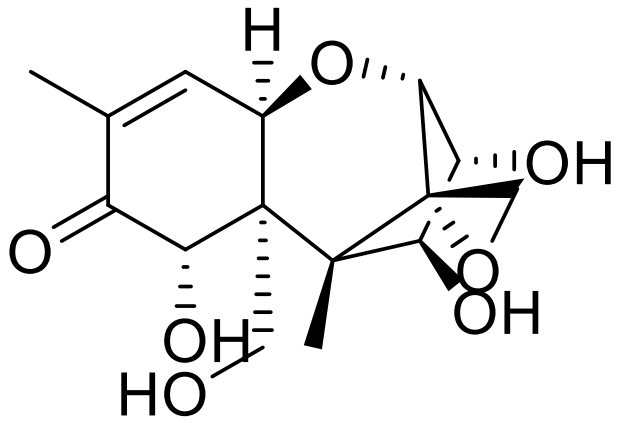

Figure 7. Chemical structure of nivalenol (NIV).
(Adopted from https://chem.nlm.nih.gov/chemidplus/rn/23282-20-4).

2.2.4.3. Fusarenon-X

Fusarenon-X (FUS-X) is produced by a number of species of *Fusarium* and is chemically allocated as a type B trichothecene (Desjardins et al.,2006). FUS-X is found primarily in cereals and co-occurs at lesser concentrations along with DON and NIV. However, it has been suggested that the toxicity of FUS-X toxicity is greater than other B-trichothecenes. Its molecular structure

has the attributes of a hydroxyl groups at C-3, 7, 15, as well as an acetyloxy faction at the C-4 position (Figure 8) (Desjardins et al., 2006).

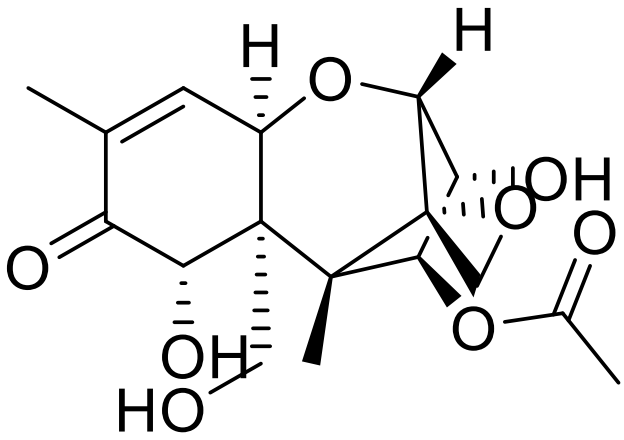

Figure 8. Chemical structure of fusarenon-X.
(Adapted from https://chem.nlm.nih.gov/chemidplus/name/startswith/fusarenon-x).

2.2.5. Type A Trichothecenes

Type A trichothecenes include compounds that have a hydroxyl group at C-8 (e.g., neosolaniol), an ester function at C-8 (e.g., T-2 toxin), or no oxygen substitution at C-8 (e.g., trichodermin, 4,15-diacetoxyscirpenol, and harzianum A) (McCormick et al., 2011) (Figure 9). This is opposed to the keto function found at C-8 in type B trichothecenes.

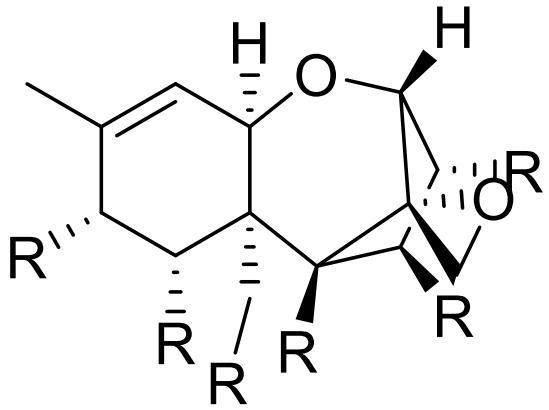

Figure 9. The general structure of type A trichothecenes.
(Adapted from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2662451/).

2.2.5.1. T-2 and HT-2 toxins

T-2 and HT-2 toxins are produced by several *Fusarium* species, *including F. sporotrichoides, F equiseti*, and *F. acumninatum*. Their occurrence seems to be greater in Europe than in North America, and the levels detected have been reported to range from 10 to <300 µg/kg (Jin and Schwarz 2020). The frequency at which they are detected also appears to be somewhat lower than for DON. T-2 toxin is considered one of the most acutely toxic trichothecenes and its levels in grain are strictly regulated in the EU and China (Fang et al., 2019). The USA currently has no regulatory or advisory guidelines.

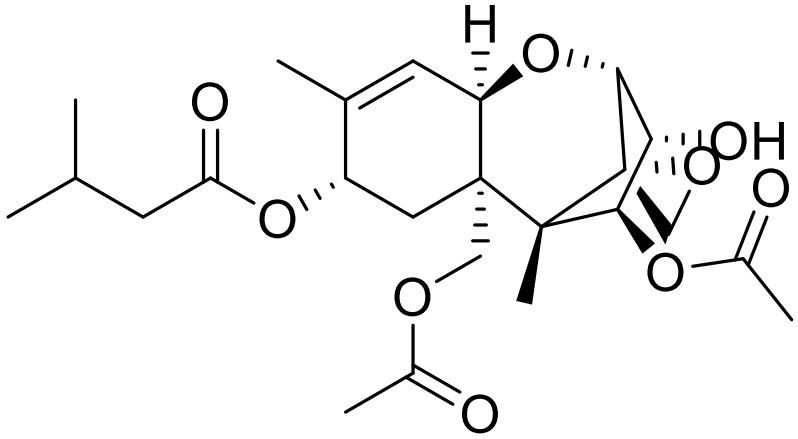

Figure 10. Chemical structure of T-2 toxin.
(Adopted from https://chem.nlm.nih.gov/chemidplus/rn/21259-20-1).

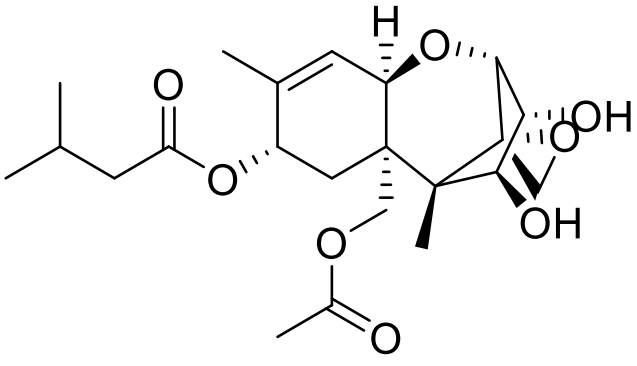

Figure 11. Chemical structure of HT-2 toxin.
(Adopted from https://chem.nlm.nih.gov/chemidplus/name/ht-2).

2.2.5.2. Neosolaniol

Neosolaniol has been reported to be produced by *F. acuminatum* and is not commonly associated with the contamination of grains (Desjardins 2006). As stated in the previous section, it is characterized by having a hydroxyl group at C-8 (Figure 12).

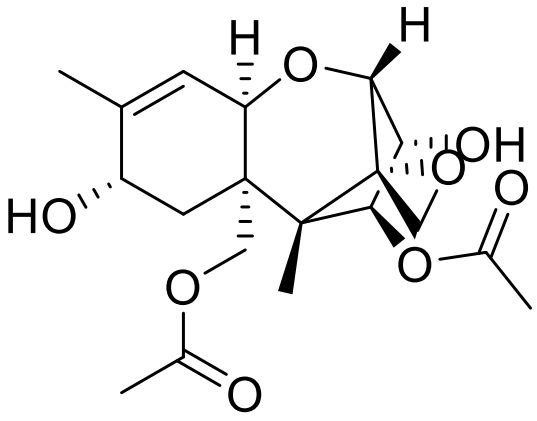

Figure 12. Chemical structure of neosolaniol.
(Adopted from https://chem.nlm.nih.gov/chemidplus/rn/36519-25-2).

2.2.5.3. Diacetoxyscirpenol (DAS)

The production of 4,15- diacetoxyscirpenol (DAS) is primarily associated with *F. acuminatum* and *F. equiseti* (Munkvold, 2017; Dejardins, 2007). Cereals can be contaminated with DAS and most accounts are on sorghum, wheat, rice, and corn (Magliano and Chulze, 2013). Levels are generally low as DAS serves largely as a precursor compound of other trichothecenes in most species.

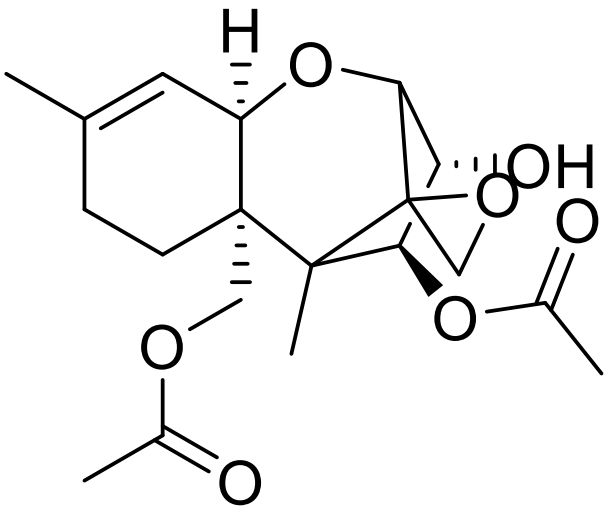

Figure 13. Chemical structure of 4,15- diacetoxyscirpenol.
(Adopted from https://chem.nlm.nih.gov/chemidplus/name/4%2C15-diacetoxyscirpenol).

2.3. Conjugated Trichothecenes

The area of conjugated mycotoxins is relatively new and they are sometimes referred to as masked mycotoxins as they often are not detected by methods routinely used in the grain trade (Berthiller et al., 2013). They form when mycotoxins are associated with polar molecules such as sugars, amino acids or lipids. Conjugation is a natural defense mechanism found in plants that increases the solubility of the toxin and aids in its transport out of the cell. While the toxicity of conjugated toxins is generally much lower than the native toxin, there is concern as masked mycotoxins may be converted to their native structures in the digestive tract of humans and animals. (Berthiller et al., 2011). Consequently, the actual amount of mycotoxins present in the food products may be miscalculated.

Most studied of the conjugated toxins is deoxynivalenol –3 – glucoside (D3G) (Figure 14), which was first reported in 2005. Subsequent study has shown it to be the major form in cereal grains, although conjugated forms of other trichothecenes and zearalenone have been reported. D3G is formed in the developing grain through the action of host plant UDP-glucosyltransferases, which catalyzes the transfer of glucose to DON. Only a portion of the total

DON is converted to D3G, and several surveys have reported the mol% of DON3G/DON to be in the range of 15-30% (Schwarz et al., 2014). A large survey conducted on maize in China showed ranges of 0.30 –4,374 and 3.00–500 µg/kg, for DON and DON3G, respectively.

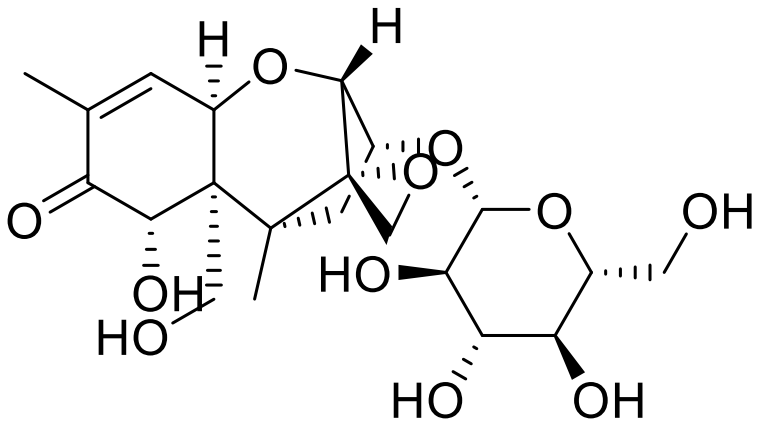

Figure 14. Chemical structure of deoxynivalenol-3-glucoside
(Adopted from https://chem.nlm.nih.gov/chemidplus/name/deoxynivalenol%203-glucoside)

2.4. Zearalenone

Zearalenone (ZEA), a macrocyclic β-resorcylic acid lactone (Figure 15), is produced by *F. graminearum* and *F. culmorum*, and often cooccurs with DON (Desjardins et al., 2007). Levels reported in grain have ranged from very low up to 1400 µg/kg, but it is detected at a much lower frequency than DON (Jin and Schwarz 2020). The production of ZEA is generally associated with higher humidity and lower temperatures. ZEA is of concern as it can induce estrogenic effects in humans and animals. Levels of ZEA are regulated in the EU, China, and several South American counties (Fang et al., 2019). According to the European Commission, the tolerable daily intake (TDI) limit for zearalenone is 0.2 µg/kg (European Commission, 2006).

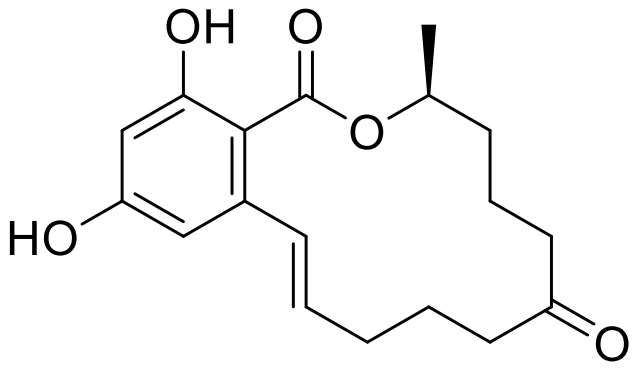

Figure 15. Chemical structure of zearalenone.
(Adopted from https://chem.nlm.nih.gov/chemidplus/name/zearalenone).

2.5. Fumonisins

Fumonisins are a group of polyketide-derived mycotoxins and are separated into four main groups: A, B, C, and P (Jin and Schwarz 2020). However, the B group includes the most toxic form, Fumonisin B1 (FB1). FB1 accounts for 70–80% of total fumonisins detected and is the cause of considerable toxicological concern. It is classified as a probable carcinogen. Fumonisins are mainly produced by *F.verticillioides* and *F. proliferatum* (Murphy et al., 2006) and have been most frequently detected in maize (Jin and Schwarz 2020). However, the presence of FB1 and FB2 was first reported in barley in 2013. In this study, conducted in Spain, fumonisins were detected in 34% of samples tested, with levels up to 300 μg/kg.

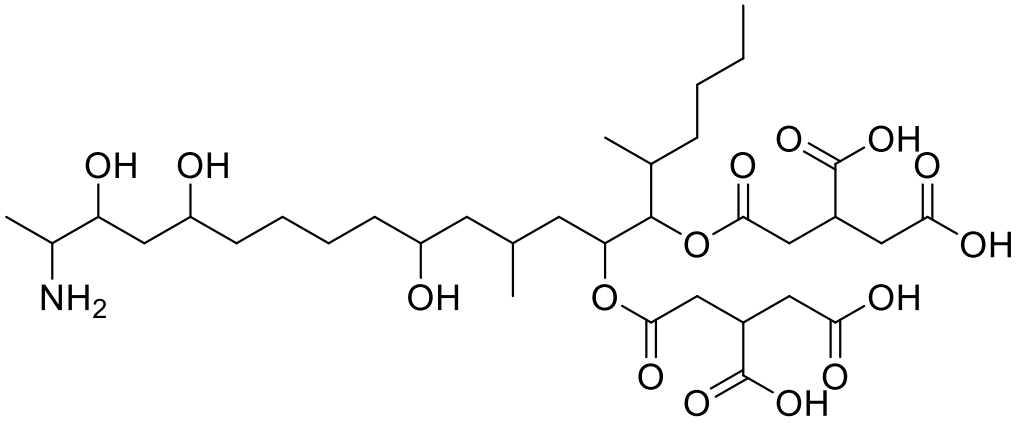

Figure 16. Chemical structure of fumonisin B1.
(Adopted from https://chem.nlm.nih.gov/chemidplus/name/fumonisin-b1).

2.6. Emerging Mycotoxins

A final area of concern in regard for *Fusarium,* is the so-called emerging mycotoxins. Emerging mycotoxins have been defined as those which are not routinely determined or legislatively regulated (Jestoi et al., 2008). Many of these have not been extensively investigated until recently (Gruber-Dorninger et al., 2017). They include the enniatins, fusaproliferin, butanolide, emodin, beauvericin, fusaric acid, moniliformin, sterigmatocystin, and tenuazonic acid among others. Within the *Fusarium* species that cause FHB, enniatins are a major emerging mycotoxin globally.

2.6.1. Enniatins

Enniatins (ENNs) consist of more than 20 different six-membered cyclic depsipeptides (Figure 17) and are mainly produced by *F. avenaceum* (Gruber-Dorninger et al., 2017). ENN occurrence is high in northern and eastern Europe on both barley and wheat (Jin and Schwarz, 2020) with incidences up to 100%. A survey conducted in Spain showed that ENNs contaminated 89%, 62%, and 50% of maize, wheat, and barley samples, respectively. It has been suggested that the ENNs are a likely major a cereal contaminant (Gruber-Dorninger et al., 2017).

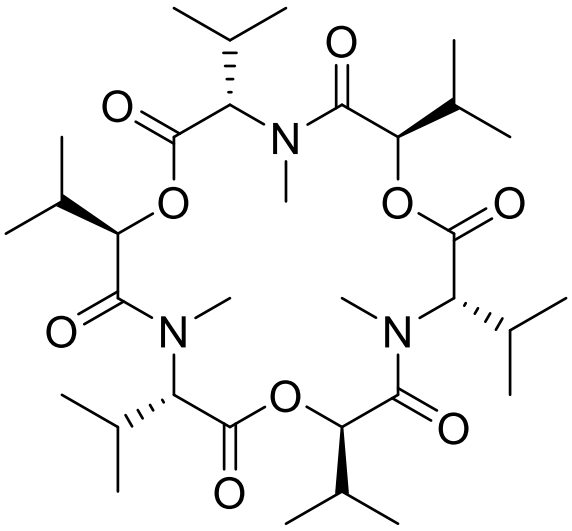

Figure 17. Chemical structure of enniatin B.
(Adopted from https://chem.nlm.nih.gov/chemidplus/name/enniatins)

2.6.2. Beauvericin

Beauvericn (BEA) is a mycotoxin of the cyclohexadepsipeptide family and is produced by *F. poae, F. avenaceum,* and *F. temperatum F. proliferatum, F. subglutinans, F. verticillioide*s, and *F. oxysporum* (Gruber-Dorninger et al., 2017). It acts as a cholesterol acyltransferase inhibitor and has been shown to be dangerous to most human cell lines. In a European survey, BEA was detected in 20, 21, and 54% of food, feed, and unprocessed grain samples collected. Maximum reported levels of BEA in grains were 6400 μg/kg. While BEA has mainly been found in maize, barley grown in both the Czech Republic and Tunisia has been reported to contain BEA, with levels up to 75 μg/kg (Jin and Schwarz 2020).

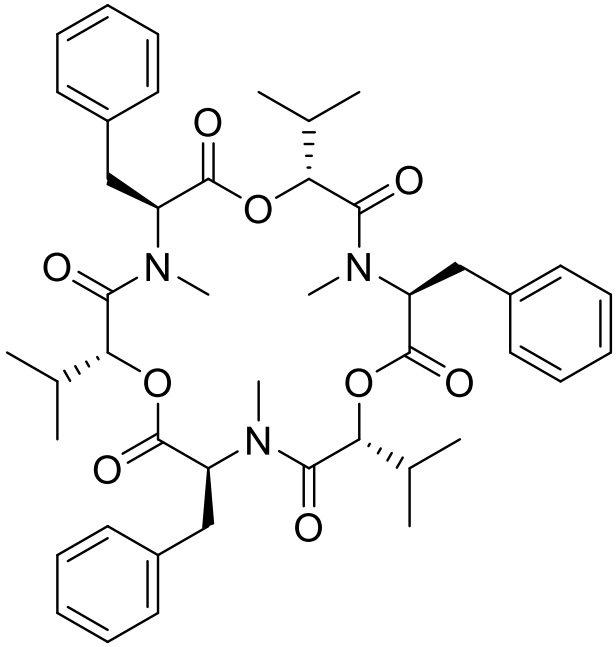

Figure 18. Chemical structure of beauvericin.
(Adopted from https://chem.nlm.nih.gov/chemidplus/name/beauvericin).

2.7. Determination of *Fusarium* Mycotoxins

With the widespread occurrence of mycotoxins in agricultural commodities and its adverse impact on food safety, economic losses, and the changing regulations surrounding mycotoxins, testing is extremely important. As mycotoxins are often found at very low

concentrations, accurate and sensitive techniques are needed (Berthiller et al., 2013). Sampling is also extremely important as mycotoxins may not be uniformly distributed within a lot, or on kernels. Failure to attain a representative sample, or to have adequate sensitivity could lead to satisfactory lots being rejected, or unacceptable consignments being permitted.

Strategies for the determination of mycotoxins have included enzyme-linked immunosorbent assays (ELISA), thin-layer chromatography (TLC), high pressure liquid chromatography (HPLC) and gas chromatography (GC). The grain trade tends to rely on rapid methods that are often in the form of test kits. While test kits are often based on ELISA, other platforms include membrane-based immunoassays, fluorescence polarization immunoassays, and fluorometric assays (Turner et al., 2009). Research and analytical testing laboratories, on the other hand, tend to utilize chromatographic methods, which are often coupled with some form of mass spectrometry (MS). A number of factors influence the selection of instrumentation and methodology and can include lower limits of detection and quantitation (LOD and LOQ), detection of multiple toxins, and speed of analysis. One must also consider that analytical scenarios are constantly improving and are driven by the progress and improvement of knowledge and expertise of researchers, and by the technical innovations in the instrumentation available. However, due to the probability that co-occurring mycotoxins are present in a food or food product, reliable, sensitive, and versatile multi-mycotoxin methods are assuming greater importance.

2.8. Chromatographic Platforms for *Fusarium* Mycotoxins

As previously mentioned, published methods for the determination of mycotoxins, including those from *Fusarium*, include thin-layer chromatography (TLC) and various forms of

liquid- and gas-chromatography. Key components of each are the separation of compounds and then identification and detection. Each platform has some advantages, but also limitations.

Thin-Layer chromatography is probably the oldest technique and is based upon the separation of compounds on a sheet of glass or plastic that has been coated with a thin layer of adsorbent material. After application of the sample mixture to the plate, a solvent/solvent mixture is drawn up into the plate via capillary action. Separation is achieved as compounds will ascend the TLC plate at different rates. It has several benefits over other chromatographic techniques with the most important being the simplicity of the tools, low cost, and minimal training needs (Berthiller et al., 2013). However, disadvantages include the fact that it can only be used for nonvolatile compounds and that it has restricted resolution capacity in terms of the number of compounds that can be separated. In addition, it cannot function in completely automated systems.

Gas chromatography (GC) has been widely used for the determination of *Fusarium* mycotoxins. Gas chromatography is based on the volatilization of the sample into a carrier gas and then separation on the stationary phase of the GC column. As such, it requires that analytes be volatile or can be derivatized to form volatile compounds. For trichothecenes, hydroxyl groups are usually derivatized to trimethylsilyl (TMS) or trifluoroacetyl (TFA) derivatives (Cigić and Prosen, 2009). The need for volatility is a limitation as some mycotoxins, such as the conjugated toxin DON-3-glucoside, cannot be made volatile. In addition, the same derivatizing agent may not be equally effective for all mycotoxins found within a sample matrix. Some toxins are also thermally liable. Detection methods have included electron capture (ECD) and various forms of mass spectrometry (MS).

Gas chromatography – electron capture detector (GC-ECD) is a form of GC that analyses halogenated compounds. The electron capture detector (ECD) captures some of the electrons of the sample as they flow through the detector, which reduces the measured current. The reduction in current is compensated and is recorded as a positive peak. This method requires a carrier gas that is low in oxygen and water and is most commonly helium. This method is especially sensitive to halogens, nitriles or nitro compounds, and organometallic compounds. It is also more sensitive than detection based on thermal conductivity, but has a limited dynamic range. Its greatest use is in the detection of halogenated compounds, where it can achieve sensitivities at the part per trillion level (ppt).

Gas chromatography – mass spectroscopy (GC-MS) can be used to analyze a broad spectrum of analytes. As the sample proceeds through the GC, different components will leave the column at different times. As they leave, they are ionized by the mass spectrometer using an ionization source. The ionized molecules are then accelerated through the mass analyzer, where they are separated based on their mass to charge ratios (m/z). There are several types of mass spectrometers that can be used. Single quadrupole systems (MS) use a single quadrupole, or mass filter, whereas a triple quadrupole (MS/MS) system contains three, one (Q2) acting as a collision cell and the other two (Q1 and Q3) acting as mass filters. MS/MS systems provide higher selectivity, better signal to noise ratios, and better accuracy at low concentrations. GC-MS struggles to analyze compounds dissolved in water because the column needs low water and oxygen to maintain resolution. Because of this, mycotoxins with high polarity can prove difficult to analyze. Mycotoxins can be quantified with the GC-MS with reasonable sensitivity at µg/kg levels of sensitivity. Today GC-MS is probably the most widely used GC format, with both single and triple quadrupole instruments being utilized routinely.

Liquid chromatographic platforms include both liquid chromatography-mass spectroscopy (LC-MS) and high-performance liquid chromatography (HPLC). In comparison to many other tandem mass spectrometry methods, LC-MS has the benefit of detection sensitivity, better resolution, and higher accuracy. In addition, its increased resolution and high accuracy present it as a better qualitative technique for fragment ions (Berthiller et al., 2018). The major disadvantage of this method is that it is costly, is non-portable, and needs an experienced technician.

High-performance liquid chromatography (HPLC) is an optional technique that offers a number of advantages, including rapid separation, the absence of the requirement for derivatization, sample stability, as well as minimal sample sizes (Berthiller et al., 2018). The disadvantage of this method is it heavily relies on variation in compound polarities, which make them susceptible to coelution. Detection can be by UV-VIS, fluorescence, or MS. Both UV-Vis and fluorescence rely on the presence of a chromophore. Some toxins, such as aflatoxins AFB2 and AFG2, are naturally fluorescent, making them much simpler to analyze using fluorescence or UV-Vis (Cigić and Prosen, 2009). *Fusarium* mycotoxins may not contain a chromophore, making detection using these methods more difficult (Turner et al., 2009). The analysis of these compounds then requires derivatization, which adds additional time and expense to sample preparation and cleanup.

LC-MS enables rapid identification of the analytes with high accuracy and reliability. Furthermore, it provides the high selectivity and simultaneous measurement of compounds at relatively low concentrations. LC-MS can involve two different methods of detection, single and tandem mass spectrometry (MS/MS). Single MS analyzes only the precursor ion, whereas tandem MS, or MS/MS, filters for the precursor ion as well as fragments of the precursor.

Benefits of MS/MS include increased sensitivity, and that more knowledge about the structure can be gained (Cigić and Prosen, 2009). The advantage of LC-MS/MS is its ability to analyze multiple analytes in samples using highly sensitive and selective MRM. In MRM methods, a parent ion will be selected in the first quadrupole (Q1MS) and then transferred into second quadrupole (Q2 MS) called collision cell, where ions accelerated with collision energy collide with nitrogen gas and produce a daughter ion. Subsequently, these daughter ions are selected in the third quadrupole (Q3MS) and last hit the detector and produce signal in the form of peak. One of the disadvantages of LC-MS/MS is low resolution (generally 0.7 Da) and limited mass range (up to 3000 m/z). It also relies heavily on the sample preparation and cleanup, which can make the analysis of certain combinations difficult or impossible without significant time or unpredictable ionization suppression.

Liquid chromatography –quadrupole-time of flight mass spectrometry (LC-TOFMS) is a derivative of mass spectrometry analysis that uses an ion's time of flight measurements to determine its mass. This is done by accelerating the ion using an electric field of known strength. The resulting velocity will depend on the mass-to-charge ratio. This method boasts increased mass accuracy and resolution, increased sensitivity, and increased dynamic range over a broad molecular weight range when compared to single quadrupole and triple quadrupole. This method has seen limited use in the detection of mycotoxins but was used to screen mycotoxins quickly without the use of reference standards (Castillo et al., 2016). LC-QTOFMS is limited by its inability to reach the lowest levels of sensitivity. When compared to a triple quadrupole running MRM methods, tandem mass spectrometry provides lower detection limits, but QTOF is able to separate potential interferences as well as provide better insight in the potential molecular formula and structure (Geib et al., 2016).

2.9. Multi- Mycotoxin Methods

As more and more toxins are identified and, in some cases, regulated, the determination of multiple toxins in a single analysis is becoming increasingly important. While methods developed in the past where often capable of measuring a number of toxins, the range was often limited, and often to toxins within the same class. For example, the GC-ECD method of Tacke and Casper (1996) measured DON, 3-ADON and 15-ADON, as well as NIV at levels from 0.2 to 40 mg/kg. The GC-MS method of Mirocha (1998) was capable of detecting DON, 15-ADON, and NIV at levels from 0.025 ng to 8 ng. The sample was extracted using acetonitrile/water (84/16 v/v), and Mirocha reported a 1 mg/kg recovery of 97.2%, displaying the sensitivity and accuracy of this method.

However, the use of GC for multi-toxin analysis is limited to those that can be made volatile and are not heat labile. Today, a broad spectrum of toxins of interest might include those from several classes, such as trichothecenes, fumonisins, aflatoxins, ZEA, ochratoxins, etc.(Mirocha et al., 1998). Conjugated toxins, such as DON3G have also become of increased interest. The higher molecular mass of these compounds can present problems for GC.

While there may be no single multi-toxin method that stands out above all others, liquid chromatography connected with mass spectroscopy is probably most prevalent (Berthiller et al., 2018). For example, Varga and coworkers (2012) developed a UPLC-MS/MS for the simultaneous determination of 11 regulated mycotoxins in maize. Analytes included DON, AFB1, AFB2, AFG1, AFG2, FB1, FB2, HT-2, OTA, T-2, and ZEN. They used an acetonitrile/water/formic acid (80/19.9/0.1 v/v/v) solvent to extract the samples. Varga and coworkers used a stable isotope dilution assay method for the internal standard. Then, using a ZORBAX RRHD Eclipse Plus C18 column, they analyzed the samples using a triple quadrupole

mass spectrometer. Their results showed LODs as low as 0.04 μg/kg and LOQs as low as 0.1 μg/kg with extraction between 88 and 105% when adjusted using their internal calibration.

2.10. Sample Preparation

Sample preparation and extraction are the most time-consuming steps in the analytical process. However, these steps are critical in the success of an analytical approach. In addition to analyte extraction, analyte enrichment and cleanup can help eliminate matrix interference and help preconcentrate the analyte. One possible sample enrichment or cleanup technique is QuEChERS (Quick, Easy, Cheap, Effective, rugged, and Safe). This method homogenizes the sample and then extracts the mycotoxins using a mixture of acetonitrile and water followed by addition of salts such as NaCl and MgSo4, dispersive -solid phase extraction (D-SPE) cleanup, and primary secondary amines (PSA) . This removes the water and other unwanted co-extractives and allows the sample to be processed quickly and the method can be altered to ensure sufficient extraction of compounds, such as, pH-dependent analytes(Desmarchelier et al., 2014).

Solvent, or liquid-liquid, extraction is based on the different solubility of the analyte in two immiscible solvents. When equilibrium between the two phases is reached, the amount of solvent must be reduced, and the analyte needs to be concentrated by nitrogen evaporation. In general, this method is simple and easy to perform with standard laboratory equipment. However, it is in decline, as when compared to new techniques, as it is labor intensive because multiple extractions are necessary and substantial volumes of organic solvents are used. Most commonly, acetonitrile and water are used in the extraction of mycotoxins (Varga et al., 2012). Also, some analyte losses can occur, due to adsorption to glassware. The use of stable isotope labelling prior to extraction can help compensate for the losses of the analyte during extraction.

Most commonly, the stable isotope is added after the extraction process which only compensates for the matrix effects.

It is not possible to measure solid samples using LC or GC systems. Because of this, the sample must go through several steps prior to analysis (Varga et al., 2012). Solvent and sample need to be mixed. The two most commonly used techniques are blending and shaking. Special care must be taken to ensure that the entire sample is in intimate contact with the extraction solvent. This process has resulted in considerable improvements in the development of immunoaffinity columns and solid-phase extraction cartridges. These procedures offer the possibility of automatization and lower consumption of solvent.

A wide variety of columns are employed today. Most commonly used are silica, alumina, diatomaceous earth, and porous polymer columns. Of these, silica and modified silica are most prevalent (He et al., 2016). A wide variety of widths, lengths, and stationary phases are commercially available. In addition to these columns, immunoaffinity columns can also be used. Immunoaffinity columns (IAC) contain antibodies that are attached to an inert support material. These columns have simplified the mycotoxin analysis. They are highly selective, and very pure final solutions can be achieved. Also, immunoaffinity columns consume much less solvent than traditional methods and can be automated. Commercial immunoaffinity columns are available for aflatoxins, fumonisins, ochratoxins, DON, type A trichothecenes (T-2 toxin) and ZON. These columns contain a solid phase (e.g., agarose bed) to which antibodies targeting mycotoxin are covalently coupled (He et al., 2016). The toxin in the sample is bound particularly to the corresponding immobilized antibody. An additional advantage is that the recoveries are higher than for liquid-liquid partitioning techniques. The disadvantages of the immunoaffinity columns are limited capacity, operability being limited to a specific pH range, being single use, and the

higher price. Solid-phase extraction cartridges contain individual packings with various surface chemistries that allow a more rapid clean-up and consume fewer solvents. Silica gel and RP-18 bonded silica columns are often used because they are pressure resistant and give reproducible results.

3. OBJECTIVES

The purpose of the project was to develop an accurate quantitative multi-toxin method utilizing the Agilent 6540 UHD LC-QTOF instrument. Goals were the rapid measurement of low levels of *Fusarium* toxins such as DON, D3G,15-ADON, NIV, NEO, DAS, FUS-X, HT-2, T-2, and ZEN using the TOF-MS full scan, and optimization of simple and easy sample extraction protocol.

1. To develop a highly sensitive LC-QTOF method for quantitative analysis of 11 toxicologically important mycotoxins in *Fusarium* infected barley and wheat.
2. Achieve separation of the isomers; 3-ADON and 15-ADON.
3. Maximize sensitivity without the use of immunoaffinity or SPE cleanup.
4. To achieve detection limits <100 μg/kg for all mycotoxins.
5. Develop methods for simple and economical sample preparation.

4. MATERIALS AND METHODS

4.1. Materials

4.1.1. Wheat and Barley Samples

In this study, grain samples with no detectable *Fusarium* toxins were used for method development and were considered as a blank matrix. Wheat (n=125) and barley samples (n=125) were obtained from the NDSU Wheat and Barley Quality labs in the Department of Plant Sciences. In order to determine the absence of mycotoxins samples were screened for Fusarium mycotoxins by LC-QTOF MS (Jin et al., 2018). The resultant chromatograms were investigated for the presence of the mycotoxins in comparison with standard curves ranging from 1.56 to 100 µg/kg. Samples which showed no signal for the mycotoxins were selected as blank matrix for further study. In the end, blank wheat (n=10) and barley (n=6) samples were bulked into single samples of wheat and barley. Mycotoxin standards were spiked onto ground grain at different concentrations, as will be detailed in a subsequent section.

4.1.2. Chemicals and Standards

Biopure mycotoxin standards of NIV, DON, D3G, 15-ADON, 3-ADON, FUS X, DAS, HT-2, T-2 and ZEN were obtained from Romer Labs (Newark, DE, USA). Acetic acid (HPLC-grade) acetic acid was purchased from VWR (Muskegon, MI, USA). Ammonium formate (HPLC-grade), was purchased from VWR (Muskegon, MI, USA). Ominisolv (LCMS-grade) methanol, water and acetonitrile, were obtained from VWR (Billerica, MA, USA). Nonsterile PTFE nylon membrane filter (0.20 µm) was purchased from Membrane Solutions (Auburn, WA, USA). Ammonium fluoride 98% was obtained from Sterm Chemical Inc (Newburyport, MA, USA).

4.2. Overview of Methodology for the Determination of Fusarium Toxins using QTOF MS

LC-QTOF based analytical methods have become a powerful and state-of-the-art technique in the qualitative and quantitative analysis over the last decade. High-resolution mass spectrometry platform based analytical methods offer highly selective and sensitive quantification of mycotoxins in the complex matrices like wheat and barley. High-resolution mass spectrometry efficiently separates the analytes of interest from closely spaced interferences from the matrix. In this study, a LC-QTOF MS based method was developed for the quantitation of mycotoxins and is outlined in Figure 19. The project was divided into three main portions: (1) the preparation of standards and samples, (2) method development, and (3) method validation. Method validation involved optimization of HPLC parameters, including column and mobile phase selection. ESI sources parameters were then optimized. Validation of the method looked at specificity, the range of linearity, limits of detection and determination, and finally recovery.

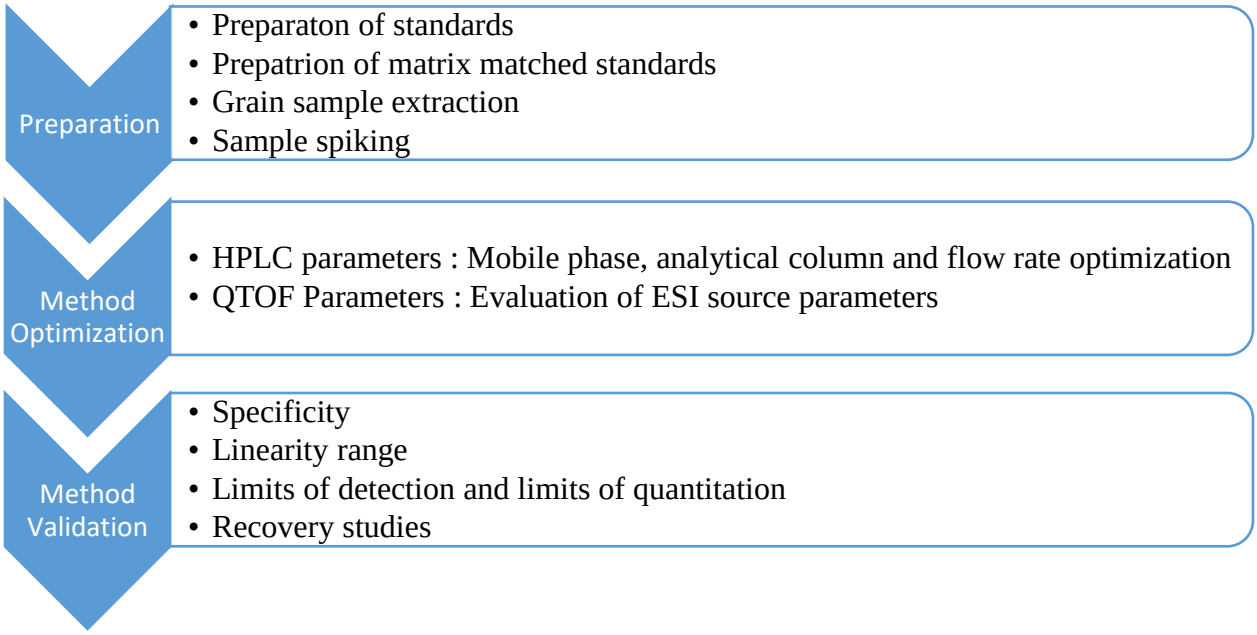

Figure 19. Outline of the experimental steps for the development of a LC-QTOF method for the determination of Fusarium toxins.

4.3. Methods

4.3.1. Preparation of Mycotoxin Standard Stock Solution

The stock solutions of individual mycotoxins NIV(200µg/L), DON (400µg/mL), D3G(50µg/mL), 15-ADON (200µg/mL), 3-ADON (200µg/mL), FUS-X (200µg/mL), DAS (200µg/mL), HT-2 (200µg/mL), T-2 (200µg/mL) and ZEN (200µg/mL) were added to 2 mL of acetonitrile: water (84:16 v/v) to yield a final concentrations of 10 µg/mL for each toxin.

4.3.2. Grain Sample Extraction

The grain sample extraction followed the procedure of Sulyok (2006). Blank wheat and barley grains were ground using Perten 3600 laboratory disc mill (Perten Instruments. Hägersten, Sweden). Each ground sample (2.5 g) was transferred to a 50 mL polypropylene tube. Then 20 mL of extraction solvent (water, acetonitrile, and acetic acid:20:79:1, v/v) was added, and the tube was vortexed for 2 min. The samples were then placed on horizontal shaker for 1 h at 150 cycle/min. The samples were centrifuged at 1370 x g for 15 min. Supernatant solution (2 mL) was transferred to a 10 mL glass tube. Next, 2 mL of hexane was added to the supernatant and the tube was vortexed for 2 min. Then the tube was centrifuged at 1370 × g for 10 min. The upper hexane layer, which contained nonpolar compounds, was discarded. The lower layer of the solvent was further diluted by the ratio of 1:10 with mobile phase A (0.1 % acetic acid, 2.0 mM ammonium formate and 1 mM ammonium fluoride in water). The solution was filtered through a 0.02 µm nylon membrane filter and analyzed by HPLC-QTOF.

4.3.3. Preparation of Matrix Matched Calibration Curve

Matrix matching is used in analysis to compensate for matrix effects that influence analytical response. This is needed in LCMS based quantitation, as ion suppression/enhancement effects due to the matrix can significantly reduce or enhance the analyte response. In the current

study, the standard solutions were added to the blank grain extracts to make the matrix matched calibration curve. Barley or wheat extracts were prepared by the extraction protocol as described in the grain sample extraction (section 4.3.2.). The protocol was the same until the hexane washing step. The hexane layer was discarded, and the lower layer of the blank extract was used for spiking.

The mycotoxin standards were spiked into blank extracted solvent (1 ml) to yield a concentration of 1000 µg/mL. Serial dilutions were then made with the same matrix extracted solvent to achieve concentrations of 500, 250, 125, 62.5, 31.2, and 15 µg/mL. Each standard prepared in the matrix extracted solvent (100 µL) was further diluted with the 900 µL of the diluent; 5 mM ammonium formate containing 0.1% (v/v) acetic acid, to make final matrix matched calibration standard concentrations ranging from 100, 50, 25, 12.5, 6.25, 3.12, and 1.56 µg/L (Table 1). The solution was filtered through a 0.02 µm nylon membrane filter prior to injecting onto HPLC-QTOF.

Table 1. Preparation of Matrix Matched Calibration Standards

Concentration of Mycotoxin Standard Prepared in the Matrix Extracted Solvent (µg/L)	**Amount Taken (µL)**	**Mobile Phase A (0.1 % Acetic Acid, 2.0 mM Ammonium formate and 1 mM Ammonium Fluoride in Water)**	**Final Concentration in Vial (µg/L)**
1000.0	100	900	100.0
500.0	100	900	50.0
250.0	100	900	25.0
125	100	900	12.5
62.5	100	900	6.25
31.2	100	900	3.12
15.0	100	900	1.56

4.3.4. Grain Spiking and Recovery Studies

The blank wheat and barley sample were ground using the Perten 3600 mill, and the flour samples were used to perform spike recovery experiments. The experiments were performed at

two concentration levels for both wheat and barley flour. Aliquots of the 10 µg/mL mycotoxin standards, (either 11.5 µL or 62.5 µL) were separately pipetted onto 2.5 g of wheat or barley flour to achieve the spiking concentrations of 46 µg/kg and 250 µg/kg. The extraction protocol was followed as described in the grain sample extraction, section 4.3.4. These extracted samples were analyzed by LC-QTOF MS. The area counts obtained for these spiked samples were used to calculate the spike recovery using the calculation described below (He et al., 2018).

Spike recovery = Area count of the spike sample / Area count of the matrix matched standard of the same concentration*100

4.3.5. Evaluation of Chromatographic Parameters

As part of method development, a number of reverse phase columns and mobile phase parameters were investigated. Columns tested are shown in Table 2.

Table 2. Columns Evaluated for Separation of Fusarium Toxins

Column	Source	Phase	Pore size (µm)	Size (mm)
Acquity UPLC HSS cyano	Waters. Milford, MA	Reverse phase	1.8	100 x 2.1
Agilent Zorbax C18	Agilent,Santa Clara,CA	Reverse phase	1.8	50 x 2.1
Agilent Pursuit PFP	Agilent,Santa Clara,CA	Reverse phase	2.7	150 x 2.1

Each column was evaluated with a number of mobile phase combinations (methanol, acetonitrile and water) and with different combinations of modifiers such as ammonium acetate, ammonium formate, ammonium fluoride acetic acid and formic acid. Combinations of parameters evaluated are shown in Table 3. In addition, the impact of flow was evaluated at flow rates of 0.2, 0.3, 0.4, and 0.5mL/min. The overall objectives were to achieve best possible separation from the matrix components and the highest sensitivity.

Table 3. Mobile Phase Combinations Used in Evaluation of Columns for the Determination of Fusarium Toxins

Mobile Phase Parameters (binary solvent components: A, B)	Column			Gradient	
	C18	PFP	HSS CN	Time (min)	% B
A. Water with 0.1 % formic acid	✓	✕	✕	0−0	10%
B. Acetonitrile with 0.1 % formic acid				2.0	10%
				3.0	20%
				7.0	24%
				10.5	30%
				13.5	60%
				15.0	70%
				18.0	75%
				18.1	95%
				21.9	95%
				22.0	10%
A. 1 mM (NH4F), 2mM ammonium formate (NH4HCO2) in 0.1% acetic acid (AA) in water	✓	✓	✓	Gradient used for final method development is detailed in section 4.3.5.	
B. Methanol with 0.1% acetic acid					

✓ Included in the trial combination ✕ Not included in the trial combination % Percentage

After the evaluation of column performance, an Agilent 1290 series liquid chromatography system with reverse phase Acquity UPLC HSS cyano column was used for the subsequent separation of *Fusarium* toxins. For this column, the mobile phase contained solvent A (0.1 % acetic acid, 2.0 mM ammonium formate and 1 mM ammonium fluoride in water) and solvent B (0.1 % acetic acid in methanol) with the following gradient: 0−1 min (A: 95%, B: 5%), 1−1.10 min (A: 95%, B: 5%), 1.10−5 min (A: 90%, B: 10%),8−11 min (A: 60%, B: 40%), 11−12 min (A: 30%, B: 70%), 13−14 min (A: 25%, B: 75%), 14−16 min (A: 0%, B: 100%) and 16−17 min (A: 95%, B: 5%). The mobile phase flow rate was setup 0.3 mL/min, with a total run

time of 17 min. The HPLC column temperature was 30 °C. The injection volume was 20 µL, and needle wash solvent was acetonitrile: water: isopropanol (60:20:20, v/v/v).

4.3.6. Evaluation of Mass Spectrometer Parameters

Analysis were performed on an Agilent 1290 series liquid chromatography coupled with Agilent G6540 UHD Accurate-Mass Quadrupole Time-of-Flight (Q-TOF) mass spectrometer (Agilent Technologies, Santa Clara, CA). As part of method development, a number of mass spectrometer parameters were evaluated. These included fragmenter voltage, drying gas, and capillary voltage. The ESI source was first monitored in positive and negative ionization mode. After determining that the highest signals were in ESI positive mode. The fragmenter voltage was tested at 50, 90, 100 and 200 volts (V) and better intensity was observed with 100V. Next, the drying gas temperature was evaluated at 250°C and 300°C and set to 300°C. The drying flow rate was 10 L/min. The nebulizer gas was set as 35psig. The sheath gas temperature was set to 325 °C with sheath gas flow 10 L/min flow. Then, capillary voltage tested at 1000V, 1500V, 2000, 3000 and 4000V. 4000V provided a better signal over the evaluated voltage. At last, nozzle voltage was set to 500V.

The acquired data sets were processed by using MassHunter Qualitative Analysis B.05.00 software (Agilent Technology, Wilmington, USA) to identify the compounds by using the software's features like Find-by-Formula and Molecular Feature Extractor. This feature uses the acquired TOF-MS and MS/MS data to detect the compounds, confirm targets, or to identify unknowns.

The data sets were processed with Agilent MassHunter Quantitative Analysis B.05.00 (Agilent Technology, Wilmington, USA) to generate the calibration curve for the standards and

to extrapolate the unknown samples, under the standard calibration curve, to determine the levels of the mycotoxins present in the sample.

The limit of detection (LOD) and limit of quantitation (LOQ) are calculated using signal to noise ratios (S/N) of the standard analytes (He et al., 2018). If the ratio of the analyte signal is 3 times greater than that of the noise, it is considered as the LOD. The lowest concentration of standard analytes present in the matrix matched calibration curve is considered as LOQ and it has a signal to noise ratio greater than 10.

5. RESULTS AND DISCUSSION

5.1. Parameters Evaluated in Method Development

5.1.1. Mobile Phase Optimization and Column Selection

Proper selection of a method depends upon the nature of the sample. This includes its ionic nature (ionic, ionizable, or neutral), molecular weight, and solubility. The mycotoxins evaluated in the present study were polar in nature, and therefore, reverse phase chromatography was selected. Experimental parameters evaluated included column choice (Table 2), mobile phase selection (Table 3), and general chromatographic condition (flow rate and buffers). In order to achieve optimized chromatographic conditions, a number of trials were conducted.

In the first trial, an Agilent Zorbax C18 was utilized. This is the column that had been routinely used in the laboratory for the determination of DON3G (Simsek et al.,2013). The mobile phase was evaluated with solvent A being water with 0.1 % formic acid, and solvent B being acetonitrile with 0.1% formic acid. For this trial, the gradient conditions used are shown in Table 3. Mycotoxin standards were injected to verify the resolution and intensity of mycotoxins. In this this trial, it was found that, the separation was achieved for all the mycotoxins except 3-ADON and 15-ADON (Figure 20). It was also observed that there was very low ion intensity for all the ions. Due to lower ion intensity, mobile phase B was changed from acetonitrile containing 0.1% formic acid to methanol containing 0.1% formic acid. This change showed improved ion intensity on Agilent Zorbax C18 column.

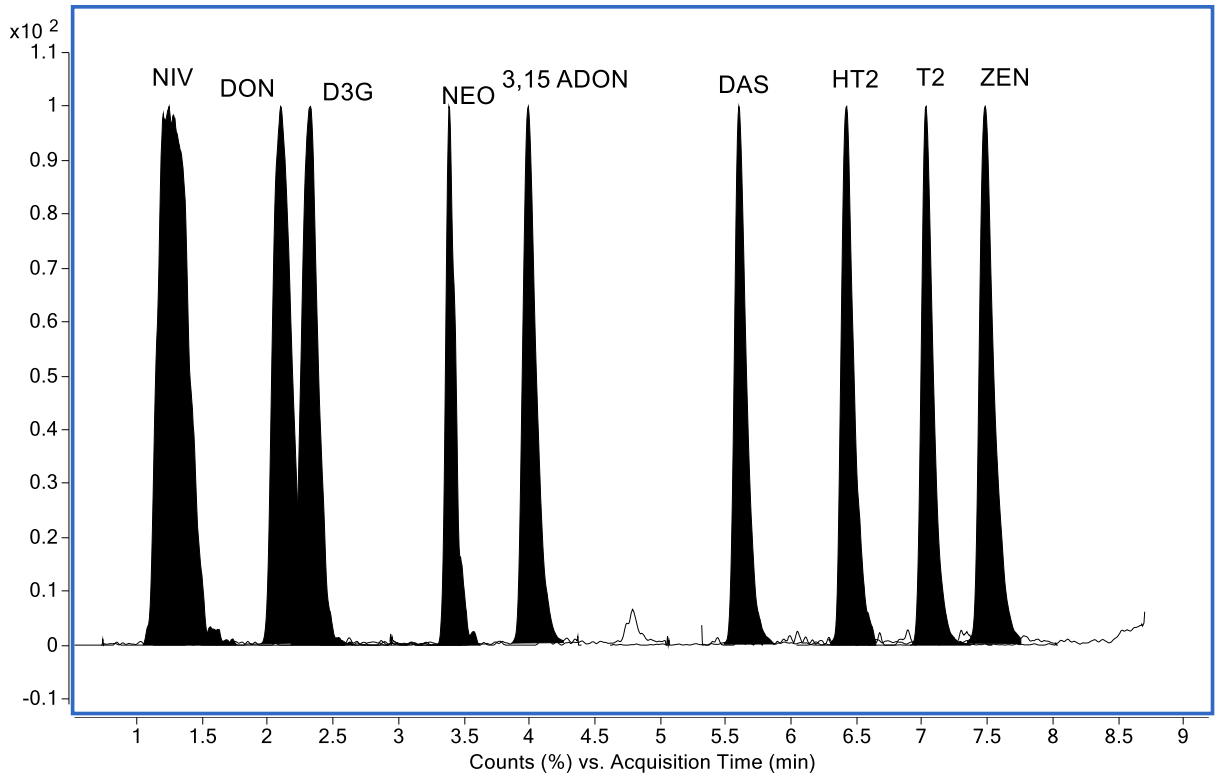

Figure 20. Representative chromatograph for 10 *Fusarium* toxins on the Agilent Zorbax C18 column using mobile phase containing solvent A. water with 0.1 % formic acid, and solvent B being acetonitrile with 0.1% formic acid. X-axis and Y-axis show retention time, and intensity respectively.

In the second trial the same column was used, but different combinations of buffers were evaluated with water and methanol mobile phases. Mobile phase A was 1mM ammonium fluoride and 2mM ammonium formate with 0.1 % acetic acid in water (w/v %), and mobile phase B was methanol with 0.1% acetic acid. This trial showed remarkable ion intensity as compared to mobile phase water: acetonitrile containing 0.1% formic acid shown in Figure 21. However, similar profile as trial 1 was observed in terms of separation.

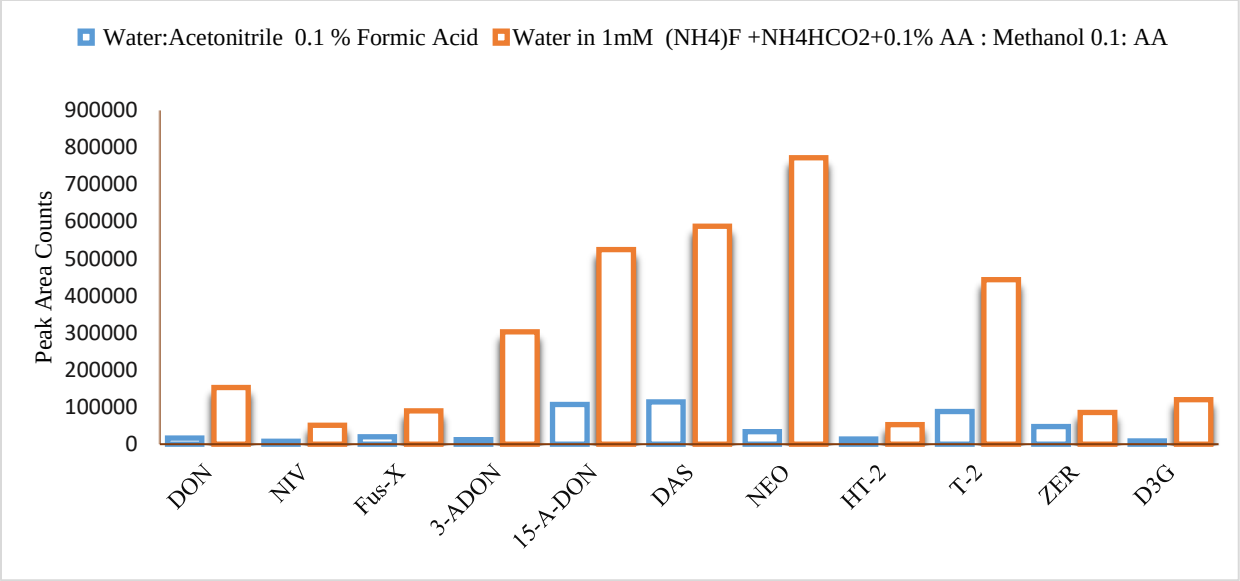

Figure 21. Peak intensity of mycotoxins separated on an Agilent C18 column as impacted by mobile phase. Mobile phase A: 1mM ammonium fluoride and 2mM ammonium formate with 0.1 % acetic acid with water (w/v %) and mobile phase B: Methanol 0.1% acetic acid. X-axis and Y-axis show analytes, and peak area count respectively

In the third trial, three analytical columns were evaluated for performance in terms of selectivity and sensitivity. Overall, the challenge was to separate the two regioisomeric compounds 3-ADON and 15-ADON. As mentioned above, trials 1 and 2 were conducted utilizing an Agilent Zorbax C18 column. While literature on mycotoxin analysis has reported the use of the C18 column the LCMS/MS method development (Jin et al., 2018), there has been no report showing separation of 3-ADON and 15-ADON on the C18 column. Figure 22 shows no separation was achieved on C18 column. This was also the case in the current study. However, this column shown better separation for all other Fusarium toxins (Figure 22).

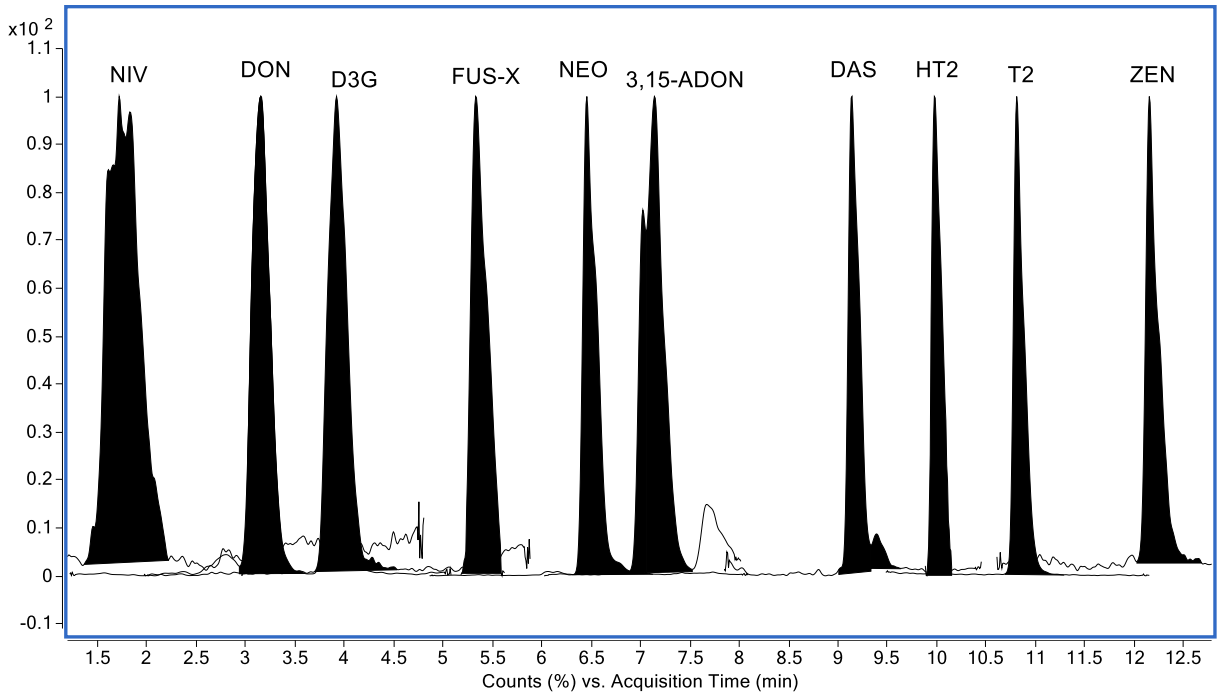

Figure 22. Representative chromatograph for 11 *Fusarium* toxins on the Agilent Zorbax C18 column, using mobile phase A. water with 0.1 % formic acid, and solvent B being methanol with 0.1% formic acid. X-axis and Y-axis show retention time, and intensity respectively.

As separation of 3-ADON and 15-ADON could not be achieved on the C18 column, additional columns were evaluated. A pentafluoro phenyl (PFP) column was tested with 1 mM ammonium fluoride (NH4F), 2mM ammonium formate (NH4HCO2) in 0.1% acetic acid (AA) in water and methanol. This experiment showed partial separation of 3-ADON and 15-ADON (Figure 23), partial separation of DON and D3G, as well as, better overall separation of the rest of the compounds (Figure 24). When this column was tested, FUS-X was not included in the standard mix. Only ten analytes are shown in the chromatogram (Figure 24). This column was evaluated as the PFP (pentafluorophenyl) stationary phase can give extra retention and selectivity for positional isomers of halogenated compounds, it can also be used for selective analysis of non-halogenated compounds, such as polar compounds containing hydroxyl, carboxyl, nitro, or other polar groups (Richheimer et al., 1994). This selectivity is enhanced when the functional groups are located on an aromatic or other rigid ring system.

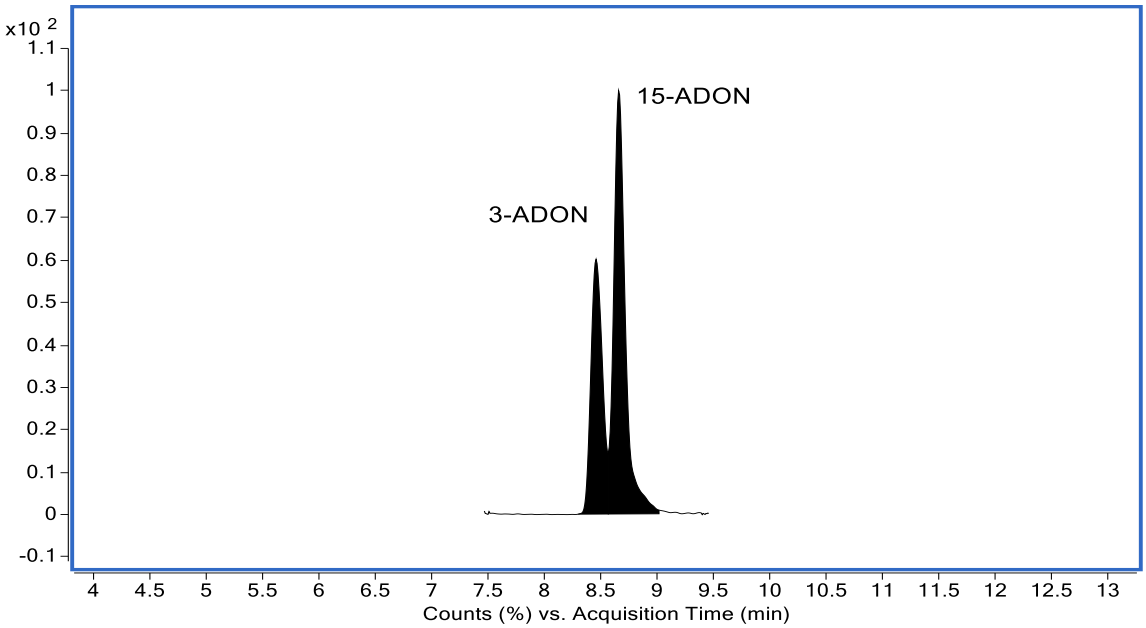

Figure 23. Representative chromatograph showing the partial separation of 3-ADON and 15-ADON on the Agilent poroshell PFP column. X- and Y-axis show retention time and intensity of the ions respectively.

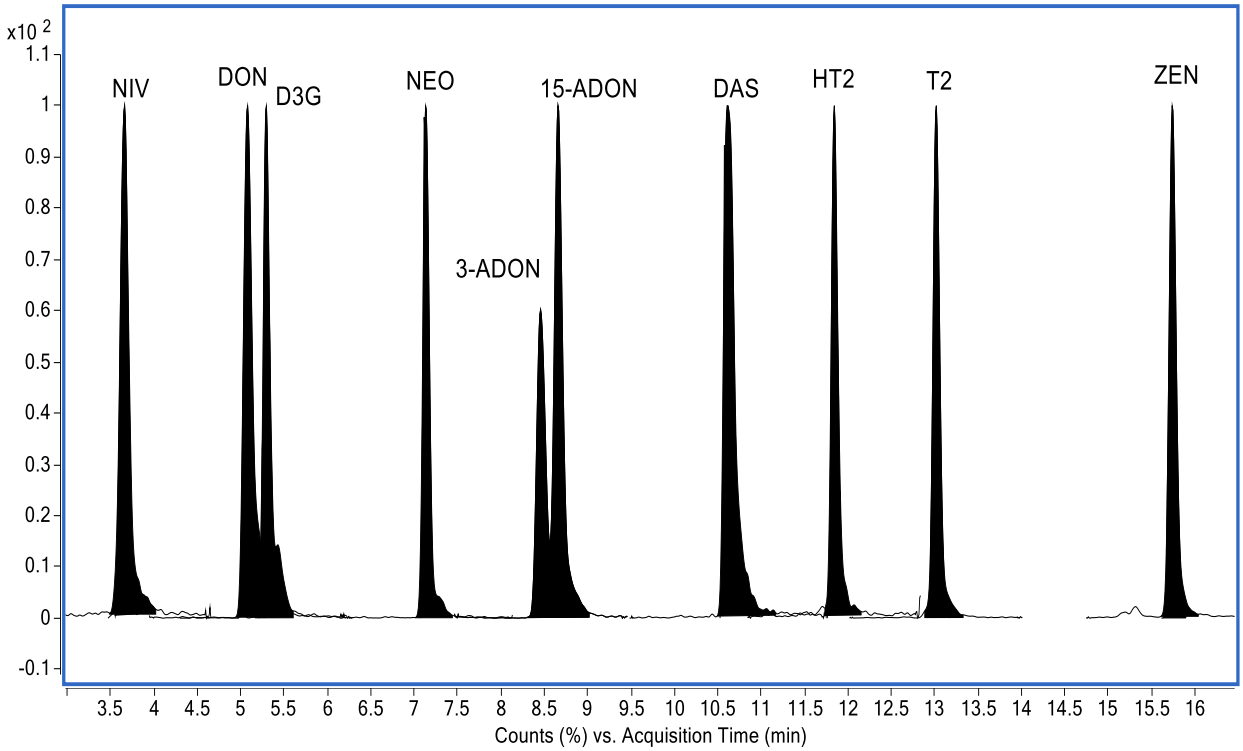

Figure 24. Representative chromatogram of 10 *Fusarium* mycotoxins on the PFP column using mobile phase 1 mM ammonium fluoride (NH4F), 2mM ammonium formate (NH4HCO2) in 0.1% acetic acid (AA) in water and methanol. The Y-axis represent ion intensity and the X-axis represents the retention time of analytes.

Finally, a Waters Acquity HSS CN (cyano propyl) column was tested. The basis for this selection was published reports that that the Waters CN column enables separation of regioisomers and stereoisomers by strong dipole movement and moderate hydrophobic nature from an alkyl ligand that can interact with other dipoles on atoms in functional groups (Henry et al., 2010). A chromatogram of the 100 µg/kg standards on the CN column is shown in Figure 26. All *Fusarium* toxins were completely separated with good peak shape, except for DON and D3G, which were only partially separated (Figure 26). This column also showed baseline separation of 3- and 15-ADON. Differences in the hydroxyl group position may have been responsible for this separation. In the case of 3-ADON, the hydroxyl groups are more proximate to each other, and may have shown stronger electrostatic interaction with the CN functional group than 15-ADON. The analytical performance of the Waters Acquity UPLC HSS cyano in terms of column intensity was much better than that of either of the Agilent Zorbax C18 or PFP columns for this study (Figure 25).

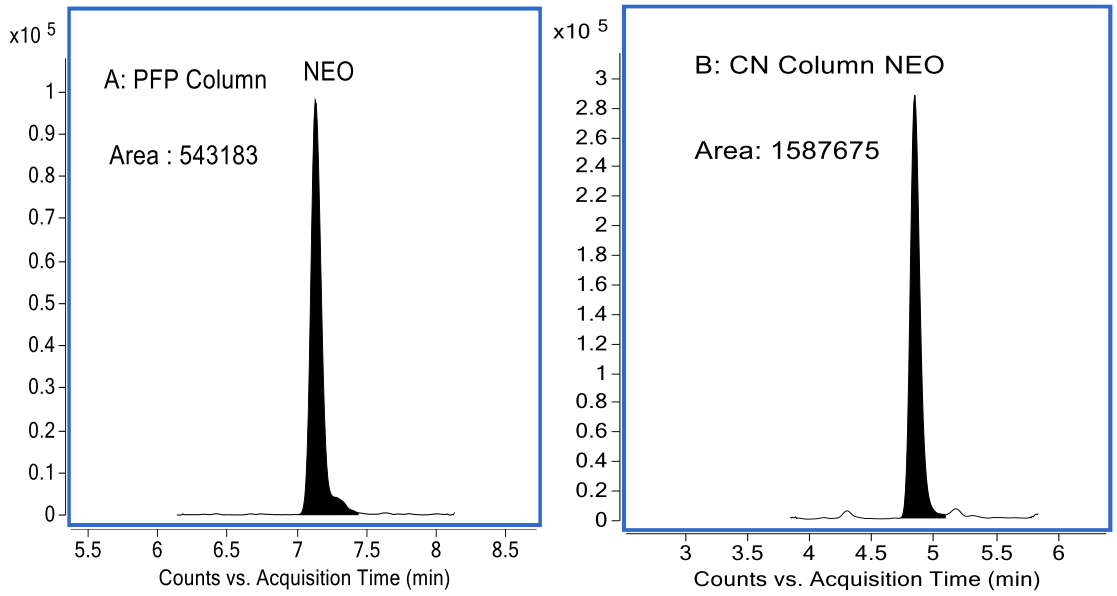

Figure 25. Extracted ion chromatogram for NEO at the concentration level of 100 µg/kg. Chromatogram A was obtained on the PFP column and B was obtained on the CN column. X-axis and Y-axes show retention time, and intensity respectively

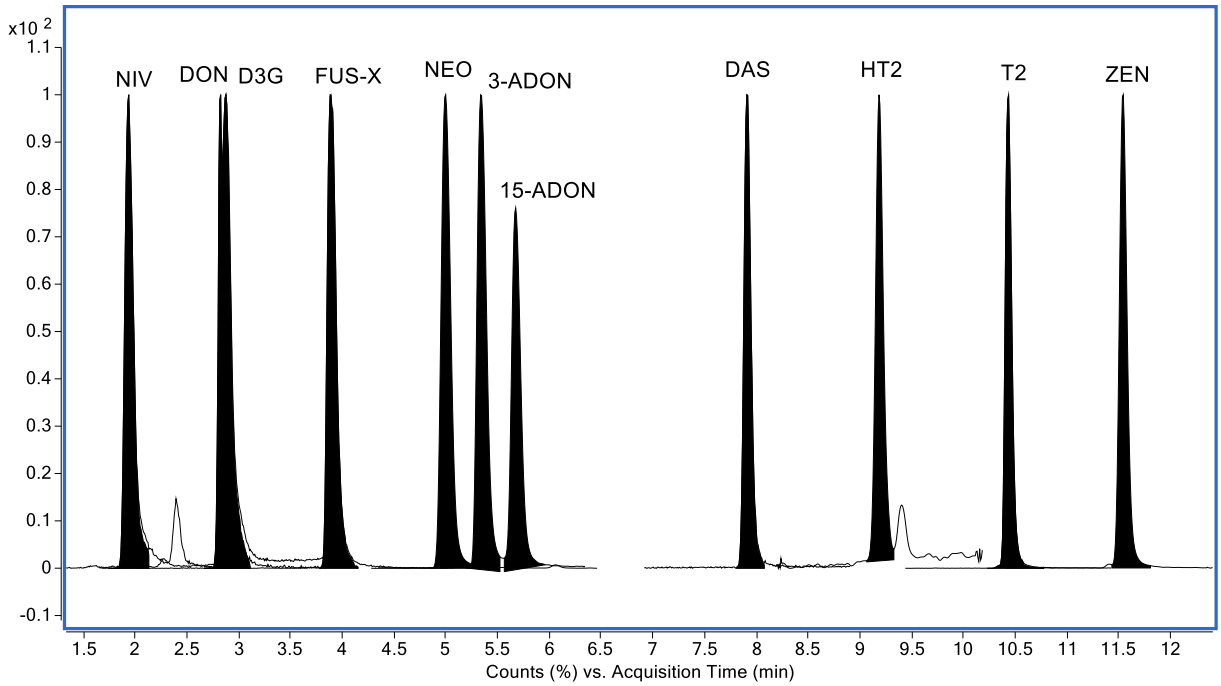

Figure 26. Representative chromatograph for 11 *Fusarium* toxins, on an Acquity HSS CN column. X- and Y-axes show retention time and intensity of ions, respectively.

All three HPLC columns were evaluated with same mobile phase at different flow rates (0.2, 0.3, 0.4, and 0.5 mL/min). Hoverer, the results were similar for flow rate with all three columns. With the CN column, the trial with the 0.2 mL/min showed broader peak widths and tailing of the peaks (Figure 27), when compared to the 0.3 flow rate. Improvement was noticed in terms of peak shape, peak intensity and separation of DON at the higher flow rate (Figure 27). Further, flow rates of 0.4 and 0.5 mL/min were explored. These resulted in a sharper peak and higher peak intensities, but also higher back pressures. Separation of 3-ADON and 15-ADON was not accomplished on the Waters CN column with higher flow rates, due to high back pressure, and these analytes merged into a single peak. As such, a flow rate 0.3 mL/min was determined to be optimal for separation and intensity of the analytes on the CN column.

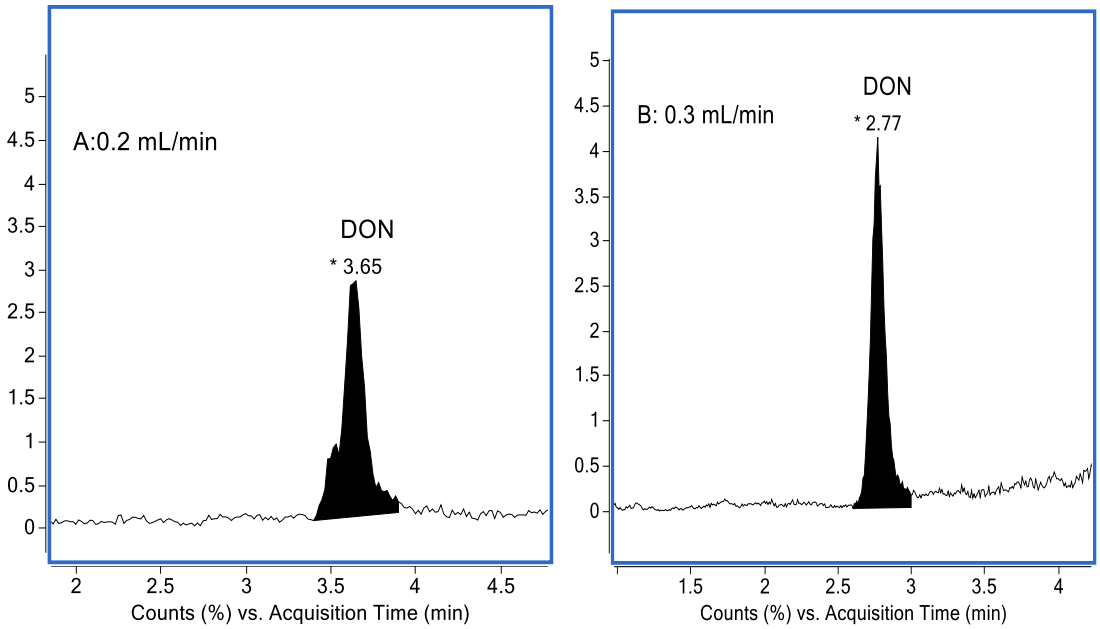

Figure 27. Representative chromatograms for DON on column Waters CN A: 0.2 mL/min flowrate. B:0.3mL/min flow rate. X- and Y-axes show retention time and intensity of ions respectively.

5.1.2. Evaluation of ESI Source Parameters

The 11 fusarium toxins standards of 1mg/kg (precursor mass of the analytes is described in Table 4.) were dissolved in the acetonitrile and injected into the HPLC without the column. The liquid flow from HPLC was introduced to the source. The first step in the source is to convert liquid droplet into a fine spray utilizing nitrogen gas. These smaller droplets had positive ions on the surface, which came under a heating gas and reduced the size of the droplet (Hofstadter et al., 1996). When the size of the droplet is reduced, the repulsion between same charge ions will increase and at certain point it overcomes the surface tension of the droplet and breaks the droplet and releases the ions into the gaseous phase. This phenomenon is called desorption/columbic explosion of ions (Hofstadter et al., 1996). The fragmentor voltage applied on the skimmer which helps to pull the ions into the mass analyzer. The capillary situated into the interface region and had voltage on it which made the potential difference segregate the ions on the basis of positive or negative voltage applied. Due to potential voltage difference ions

transfer through mass analyzer and reach the detector. In the mass analyzer ions separates according to their mass to charge ration.

In this method development, both the positive and negative modes were investigated for ion acquisition in the MS. The highest signal was observed in the ESI positive mode. The mass spectrometer was operated in positive mode. The fragmenter voltage was ramped from 50 V, 90 V, 100V and 200 V. The highest intensity was observed with 100 V. The drying gas temperature was set to 300°C with a drying-gas flow rate of 10 L/min, after evaluating multiple temperature values. The sheath gas temperature was set to 325 °C with 10 L/min flow. The nebulizer (N2) was set at 35 psig, the fragmentor voltage was set to 110 V, the skimmer voltage was set at 65 V, the octupole RF voltage was set at 750 V, and the capillary voltage was evaluated at 1000 V, 1500 V, 2000 V, 3500 V and 4000 V. 4000 V provided the highest signal over the evaluated voltages. Nozzle voltage was set to 500 V. The collision energy was set as 0 eV. The TOF-MS scan mass range was set to 100 to 600 amu.

Table 4. List of Compounds Included in the Method with Their Adduct, Precursor Ion Mass, Fragmentor Voltage and Polarity.

Compound	**Adduct**	**Precursor Ion (M/Z)**	**Fragmentor Voltage(V)**	**Polarity**
NIV	M+ H	313.1269	100	Positive
DON	M+ H	296.1319	100	Positive
DON3G	M+ NH4	476.2121	100	Positive
FUS-X	M+ H	355.1592	100	Positive
15-ADON	M+ H	339.1438	100	Positive
3-ADON	M+ H	339.1438	100	Positive
T2	M+NH4	484.2535	100	Positive
HT2	M+ NH4	442.4423	100	Positive
NEO	M+H	400.1966	100	Positive
DAS	M+NH4	384.2012	100	Positive
ZEN	M+ H	319.1533	100	Positive

5.2. Method Validation

A reliable and robust analytical method requires full validation that addresses selectivity, linearity, precision, accuracy, and limits of detection (LOD) and quantitation (LOQ). The method validation was performed using the final chromatographic method and mass spectrometer parameters discussed in sections 4.3.5 and 4.3.6. Each of the method validation parameters and results are discussed in the following sections.

5.2.1. Specificity

While the terms selectivity and specificity are often used interchangeably, the term specificity generally refers to a method that produces a response for a single analyte only (He et al., 2018) . The term selectivity refers to a method that responds to many chemical analytes that may or may not be distinguished from each other. If the response is distinguishable from all other responses, the method is said to be selective. To establish the specificity and selectivity of the method, six replicates of blank barley and wheat samples were injected and evaluated for the matrix effects with the spiked sample. As shown in Figure 28, the blank samples showed no interfering peaks at the retention times of the analytes of interest. For example, Figure 28A shows no interference for NEO, but similar results were observed for all the *Fusarium* toxins tested. As such, the method enabled monitoring the response of single analytes and their specific detection over other analytes.

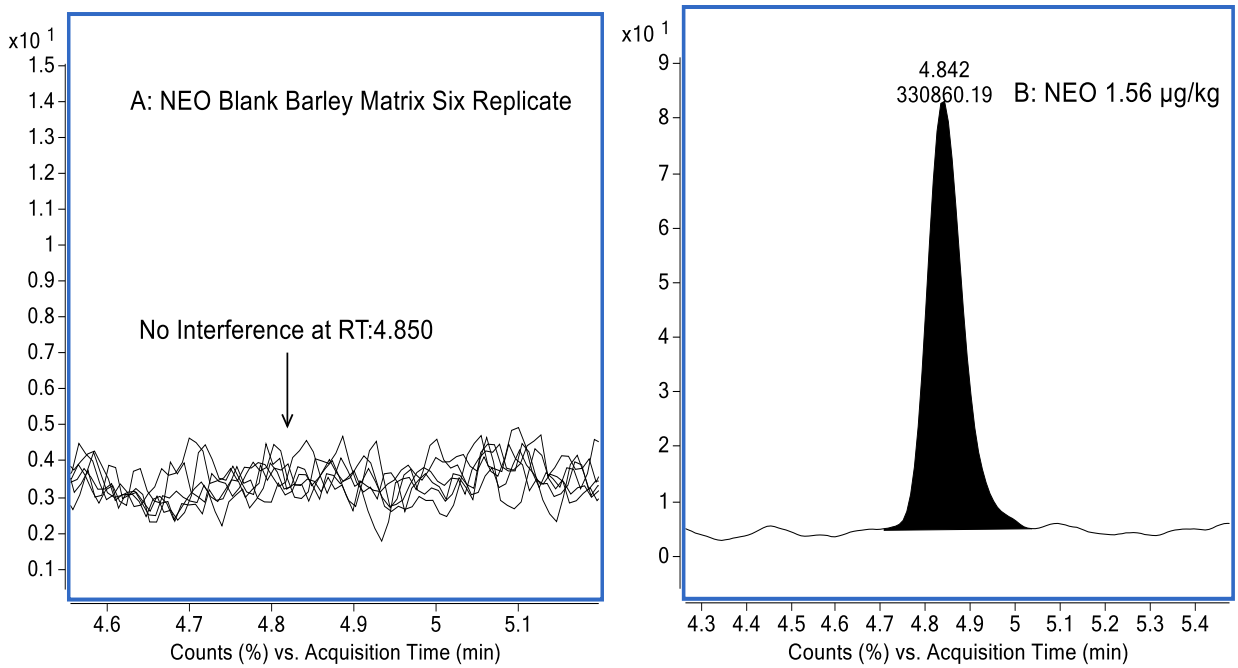

Figure 28. Chromatogram A: The six black colored lines which represent the instrument signal for each blank sample. At the retention time of 4.850 min, there no interference for the NEO blank samples. Chromatogram B: Show the peak area and retention time for NEO at 1.56 µg/kg acquired on waters cyano column.Y-axis shows the intensity and X-axis shows the retention time for the analytes.

5.2.2. Linearity Range

The range of linearity for each of the *Fusarium* toxins were determined based on the calibration graph shown in Figure 29, and data in Table 5. In the proposed method, linearity for each toxin was studied over the range of 1.5 to 100.0 µg/kg in the matrix standard solutions. Regression analysis was used to assess the linearity of the quantitation method. The calibration model was selected based on the linear regression analysis data with and without intercepts and weighting factors (1/x or 1/x2, where x is concentration). The best linear fit and the least squares were obtained with a 1/x weighting factor for all toxins. The result of a seven level (1.56-100 µg/kg) calibration in wheat and barley flour show good linearity with a correlation coefficient r = 0.9 which demonstrated excellent relationship between peak area and concentration of toxins in the tested concentration in range.

Table 5. The Linearity and Regression Results for 11 Fusarium Toxins in Barley and Wheat

Sample	Barley		Wheat	
Compound Name	Concentration Ranges (µg/kg)	R-Value	Concentration Ranges (µg/kg)	R-Value
NIV	1.56 -100	0.997	1.56 -100	0.983
DON	1.56 -100	0.998	1.56 -100	0.984
DON3G	1.56 -100	0.996	1.56 -100	0.980
FUS-X	1.56 -100	0.997	1.56 -100	0.994
15-ADON	1.56 -100	0.999	1.56 -100	0.994
3-ADON	1.56 -100	0.998	1.56 -100	0.971
NEO	1.56 -100	0.997	1.56 -100	0.995
T2	1.56 -100	0.998	1.56 -100	0.999
HT2	1.56 -100	0.999	1.56 -100	0.985
DAS	1.56 -100	0.999	1.56 -100	0.986
ZEA	1.56 -100	0.994	1.56 -100	0.998

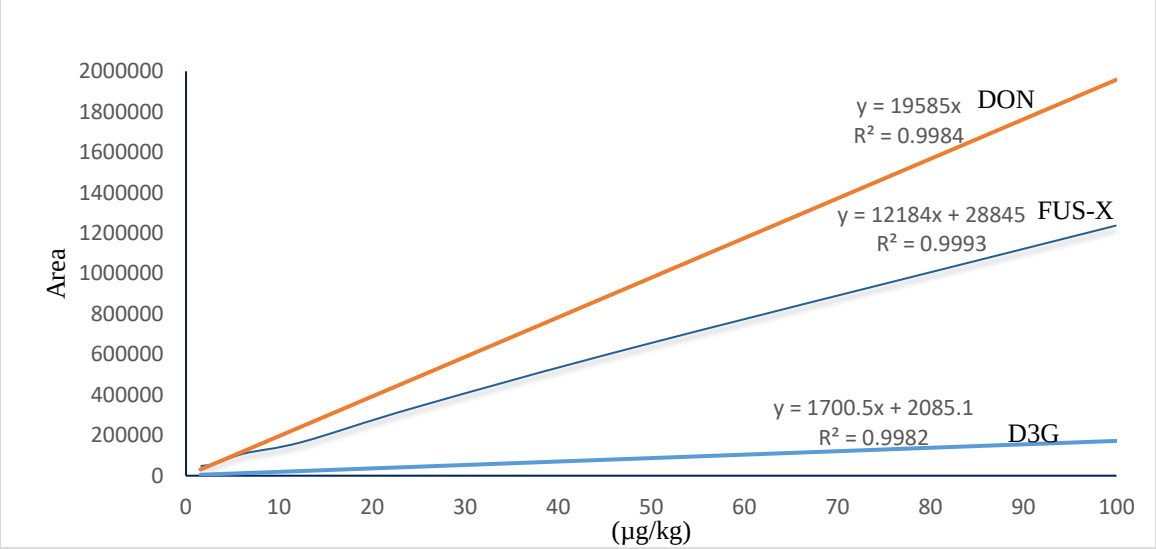

Figure 29. Example of matrix matched calibration curve for DON, D3G and FUS-X in the barley sample. Y and X axes showing the area and concentration of analytes.

5.2.3. Limits of Detection and Quantitation

The LOD and LOQ were measured based on the method described by the International Conference of Harmonization (He et al., 2018). The post-spiked standard solutions were diluted with mobile phase A to provide appropriate concentrations. LOD is defined as the lowest amount of analyte that can be detected above baseline noise. Typically, a signal to noise (S/N) ratio of 3

or above is used. LOQ is defined as the lowest amount of analyte, which can be reproducibly quantitated above the baseline noise level. Here a S/N ratio of 10 or above is typically selected.

In this study, LOD and LOQ for *Fusarium* toxins were obtained at different concentrations using the signal to noise approach, measuring chromatographic response of analytes (toxins were prepared in wheat and barley extract with two levels 0.7 and 1.56 µg/kg) and the chromatographic noise. Using this approch ,the limit of detection for *Fusarium* toxins were determined as 0.7 µg/kg (Figure 30) and the LOQ was 1.56 µg/kg (Figure 31). The calibration curve was linear and proportional, ranging from from 1.56 to 100 µg/kg. The S/N for 1.56 µg/kg is greater than 10 or fixed into the calibration curve, therefore, 1.56 µg/kg is LOQ of the method. The method showed the S/N for 0.7 µg/kg is greater than 3, thus, 0.7 µg/kg can be considered as LOD. Table 6 showed 0.7 µg/kg LOD and LOQ 1.56 µg/kg for all the 11 *Fusarium* toxins. These LODs and LOQ are relatively lower than reported by Klötzel et al., 2005 and Sulyok et al., 2008.

Sulyok (2006) designed a method on LC-MS/MS to analyze 39 mycotoxins (including DON, FUS-X, NIV, 3-ADON, 15-ADON, DAS, HT2, T2, ZEN, and NEO) in wheat and maize samples. For testing the LOD and LOQ, 0.5 gram of wheat flour taken and extracted in acetonitrile and water. In wheat samples, the LOD and LOQ values ranged from 2–16 and 10–106 µg/kg, respectively, Thus, compared to the results, these LOD and LOQ values were relatively high.

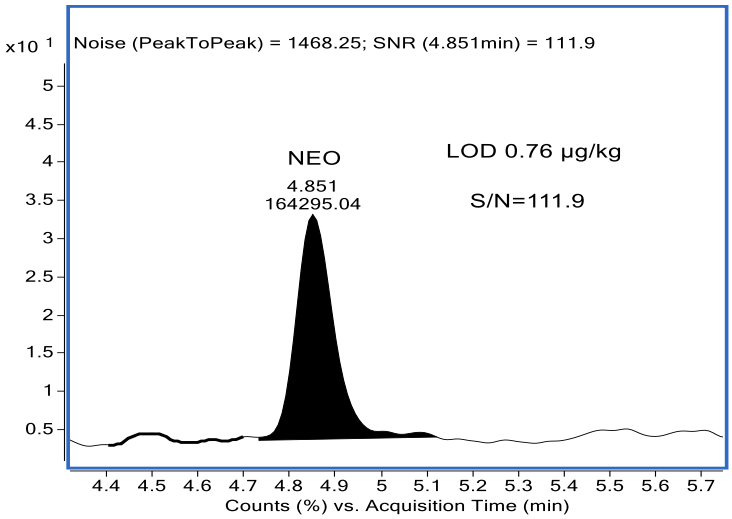

Figure 30. Determination of LOD of detection of NEO, LOD was found at 0.76 µg/kg showing S/N =111.9. X-axis and Y-axis show retention time, and intensity, respectively

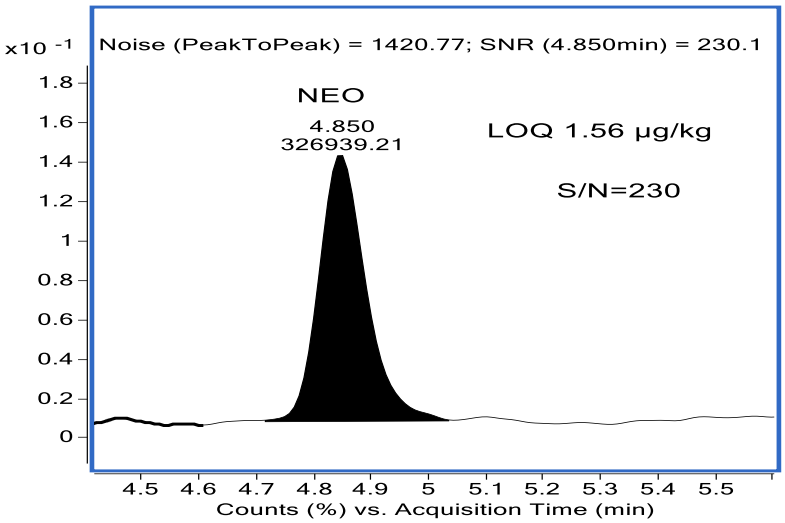

Figure 31. Determination of LOQ of detection of NEO, LOQ was found at 1.56 µg/kg showing S/N =230. X-axis and Y-axes show retention time, and intensity, respectively

5.2.4. Precision and Accuracy

The precision of a quantitative method is the degree of agreement among individual test results when the procedure is applied repetitively to multiple sampling. It is measured by repeated injections of different levels of quality control samples (He et al., 2018). Accuracy is the measurement of how close the experimental value is to the true value. It is expressed as % recovery by the assay of known/added amount of analyte in the linearity range. The inter-day and

intra-day assay precision and accuracy are presented in Table 6. Intra-day precision or repeatability was evaluated through three concentration levels (low, medium, and high) with six replicates each on the same day under the same experimental conditions.

The method was found to be accurate, precise, and reproducible for the *Fusarium* toxins from the barley and wheat samples. According to the European Commission (SANCO/12571/2013) the acceptance criteria of accuracy is within range of 70% to 120 %, and the precision percent values are within 20 %. Table 6 shows the summarized data on accuracy and precision for the 11 mycotoxins at three different levels. From all 11 *Fusarium* toxins evaluated, 11 *Fusarium* toxins demonstrated accuracy in the range of 80% to 120% and precision percent was within 20%. The achieved relative standard deviation for the peak area was between 0.9% to 8.6%. These toxins values are smaller than reported values by Klötzel et al., 2005.

Table 6. Accuracy and Precision % of *Fusarium* Toxins for the Three Spike Concentration Levels Studies in Barley.

		Intra-Day (n=6)		Inter-Day (n=6)	
Compound Name	Concentration (µg/kg)	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)
NIV	1.56	94	3.50	100.95	2.382
	12.5	107.9	7.211	104.4	6.825
	50	100.65	4.775	98.55	4.222
DON	1.56	101.5	5.25	103.75	6.789
	12.5	97.7	8.60	96.95	1.565
	50	100	0.9	100	2.268
DON3G	1.56	99.6	0.4252	96.2	1.188
	12.5	104.3	6.314	103.4	2.733
	50	100.55	3.951	99.45	4.573
FUS-X	1.56	95.9	5.655	96.1	5.170
	12.5	105.5	4.7838	108.35	1.891
	50	99.1	0.4075	98.35	1.696
3-ADON	1.56	108.6	5.262	105.7	7.181
	12.5	90.5	6.164	96.5	1.733
	50	101	6.164	100.55	1.495
15-ADON	1.56	112.5	5.262	102.85	5.123
	12.5	86.9	5.262	94.75	1.476
	50	102.2	5.39	101.65	1.625
T2	1.56	107.1	5.262	106.55	2.670
	12.5	89.5	5.26	91.65	1.602
	50	102	5.2621	101.55	0.978
HT2	1.56	100.9	6.405	106.95	5.003
	12.5	105.5	7.478	91.2	4.150
	50	99.1	5.962	102.15	1.635
NEO	1.56	96.7	5.734	95.15	3.128
	12.5	103.2	1.7569	104.45	2.183
	50	99.2	1.786	98.6	1.859
DAS	1.56	101.45	5.3007	116.4	5.858
	12.5	96.3	0.8015	94	2.432
	50	100.25	1.154	100.6	1.539
ZEN	1.56	113	5.388	114.55	2.154
	12.5	83.5	2.370	84.25	3.375
	50	105.9	5.717	107.2	7.547

5.3. Recovery Studies

Determination of the spike recoveries is an important performance parameter in analytical method validation, particularly for mass spectrometry-based analysis. As previously described, the blank wheat and barley sample was ground using Perten mill, and the flour was spiked to perfom recovery experiments. The experiments were performed at two concentration levels for both wheat and barley flour. Aliquots of the 10 µg/mL mycotoxin standards (either 11.5 µL or 62.5 µL) were separately pipetted onto 2.5 g of wheat or barley flour to achieve the final spiking concentration the sample 46 µg/kg and 250 µg/kg. The extraction protocol was followed as described in the grain sample extraction, section 4.3.4. The spike recovery percent for the different analytes ranged between 60 to 147%, with a relative standard deviation of less than 40%, in the barley and wheat samples. In the barley sample, very good recoveries for all the mycotoxins was observed. Recovery for the different analytes was between 80 and 120% with relative standard deviations (RSDs) < 20 (Table 7). In the wheat sample low recoveries for the NIV (61.88%), T2(60%), and ZEN (>60%) were observed. Better recoveries were observed for DON, D3G,3-ADON and 15-ADON respectively as described in Table 7. According to the European Commission (EU) No 519/2014, the acceptance criteria for the mycotoxin recovery is from 60% to 120% and relative standard deviation (RSD%) is < 40. However, in some cases, the percentage recovery is lower or higher than the defined acceptable range due to matrix suppression or enhancement.

Klötzel et al 2005 conducted recoveries studies in wheat samples. Type A and B trichothecenes were extracted in acetonitrile/water (84/16 v/v), and the extract was cleaned-up with a mycosep column. The recoveries ratios ranged from 51% to 95% and relative standard deviation reported ranged from 8 to 14.2%. Lower recoveries were reported for NIV (51%). The

recoveries obtained in this study are comparable with those reported by Klötzel et al 2005 and Sulyok et al 2008.

Table 7. Recoveries and Relative Standard Deviations Obtained for 11 Fusarium Toxins from Spiked Wheat and Barley Samples at the Spiking Levels of 46 µg/kg and 250 µg/kg (n = 12).

		Barley (n=12)		Wheat(n=12)	
Compound	Spiked Concentration (µg/kg)	Recovery (%)	% RSD	Recovery (%)	% RSD
NIV	46	84.00	8.48	61.88	19.61
	250	90.16	6.71	93.3	11.29
DON	46	81.94	3.67	74.20	12.17
	250	86.57	6.78	85.60	2.57
DON3G	46	84.73	8.48	73.80	3.75
	250	91.03	6.78	75.18	3.20
FUS-X	46	96.54	5.93	97.30	16.09
	250	114.88	2.06	112.1	13.89
3-ADON	46	94.82	3.30	123.42	16.50
	250	114.80	3.36	121.82	17.94
15-ADON	46	94.82	3.30	139.93	31.86
	250	114.80	3.36	147.46	20.07
NEO	46	81.066	2.04	80.4	11.83
	250	116.10	2.75	109.17	11.46
DAS	46	105.05	10.5	126.23	27.78
	250	89.20	6.92	105.37	30.54
HT2	46	105.05	10.56	79.23	26.88
	250	89.20	6.92	89.35	12.91
T2	46	96.99	3.20	61.8	40.07
	250	110.79	2.70	64.63	22.64
ZEN	46	96.67	3.67	70.58	8.88
	250	118.14	5.88	60.78	8.923

6. CONCLUSION

In this study, a multi-mycotoxins quantitative method was developed that simultaneously identifies, confirms and quantifies 11 *Fusarium* compounds in barley and wheat using an Agilent LC-QTOF. The method also has a short chromatographic run time of only 17 minutes for each sample. The TOF-MS scan method LOD is comparable with the published reports using the MRM method, which is considered the gold standard method for quantitation. In this study, the matrix matched calibration curve was linear from 1.56 to 100 µg/kg for all 11 toxins in wheat and barley sample. Additionally, this method achieved chromatographic separation of the isomeric compounds, 3- ADON and 15-ADON. Spiked samples of 46 µg/kg and 250 µg/kg concentration showed toxin recoveries ranging from 60 to 130 %.

The sensitivity of the analytes achieved in this work is sufficient for the current regulatory guidelines in accordance to European Commission (EC NO.1881/2006) *Fusarium* mycotoxin limits specified in unprocessed and processed cereals are (depending on the matrix): 200–1750 µg/kg for DON, 20–400 µg/kg for ZEN, 15–1000 µg/kg for sum of HT2 and T-2, limits have been recommended for the sum of HT2 and T-2 toxins in various matrices. However, there could be further improvement in the sensitivity by modifying the current sample preparation protocol to dispersive-solid phase extraction, which potentially could reach a lower detection limit than in the current method (Nakhajavan et al., 2020). Method optimization for sample extraction would also help in meeting the future stringent regulatory requirements.

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