SURVEY OF *CLAVICEPS PURPUREA* AND *FUSARIUM* TOXINS IN SPRING WHEAT AND FUNGICIDE EFFICACY ON ERGOT SCLEROTIA, ALKALOID CONTENT, AND SAPROPHYTIC *FUSARIUM* ASSOCIATED TOXINS

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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

DOCTOR OF PHILOSOPHY

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ABSTRACT

Fusarium head blight (FHB) and ergot of the *claviceps purpurea* (Fr.) Tul. have adverse effects on grain quality and human and animal health. Each year, Hard Red Spring (HRS) wheat samples are collected at harvest to survey the end use quality. HRS-wheat survey samples (n= 207) were obtained from four growing states (ND, SD, MN, and MT) during 2019 and 2020. Grading and non-grading factors and the presence of Deoxynivalenol (DON) and ergot alkaloids (EAs) were determined, naturally occurring mycotoxins produced by *Fusarium* species and *C. purpurea,* respectively.

Chromatography-Mass Spectrometry (GC-MS) was utilized for DON content and Reveal Q+Max test for total EAs. The mean DON contents during the 2019 and 2020 growing seasons were 1.5 ppm and 0.6 ppm, respectively. The mean for total EAs contents in 2019 and 2020 were 81.5 ppb and 180.5 ppb, respectively. Both toxins showed location had significant ($P \leq 0.05$) effect on variations within one individual year. Deoxynivalenol and EAs shared positive and significant correlations with total defects (0.257, $P<0.001$) and (0.162, $P<0.05$), respectively.

In the field experiment, four fungicides (Sphaerex, Miravis Ace, Quilt, and Priaxor) were applied on a confidential HRS wheat cytoplasmic male sterile (CMS-HRS wheat) line. After separating the ergot sclerotia samples from other contents, they were evaluated for naturally occurring saprophytic *Fusarium* toxins and EAs. The mean value for DON content in Miravis Ace (0.07 ppm) sample was significantly ($P \leq 0.05$) less than the mean value for the non-treated control and Priaxor samples (0.24 and 0.21ppm, respectively). Ergot body weight was significantly ($P \leq 0.05$) higher for the non-treated control compared to other treatments. Among fungicides, ergot body weight was significantly lower in Miravis Ace samples (39.57 g) compared to Quilt (55.83 g), Priaxor (55.04 g), and Sphaerex (54.82 g) treated samples. Total

EAs in Priaxor (244,840 ppb) samples was significantly ($P \leq 0.05$) less than the mean values found in Sphaerex and Quilt samples (359,485 ppb and 352,375 ppb, respectively). Priaxor could be a potential fungicide to control ergot body weight and total EAs production. Future studies are needed to test more fungicides' effectiveness to reduce ergot body and total EAs production.

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LIST OF ABBREVIATIONS

AACCI ..... American Association of Cereal Chemists International

ANOVA..... Analysis of Variance

ARfD ..... Acute Reference Dose

CAC..... Codex Alimentarius Commission

CGC..... Canadian Grain Commission

CMS-HRS Wheat..... Hard Red Spring Wheat Cytoplasmic Male Sterile

CWAD..... Canada Western Amber Durum

CWRS..... Canada Western Red Spring

DHV ..... Dark Hard and Vitreous

DKG ..... Dockage

DMIs ..... Demethylation Inhibitors

DMK ..... Damaged Kernels

DNS..... Dark Northern Spring

DON ..... Deoxynivalenol

EAs..... Ergot Alkaloids

EFSA ..... European Food Safety Authority

ENNS ..... Neosolaniol

ETC ..... Electron Transport Chain

FDA..... Food Drug Administration

FGIS ..... Federal Grain Inspection Service

FHB..... Fusarium Head Blight

FN..... Falling Number

FRAC ..... Fungicide Resistance Action Committee

FUX..... Fusarenon-x

GC-MS ..... Gas Chromatography-Mass Spectrometry

HRS ..... Hard Red Spring

LSD ..... Least Significance Difference

MN ..... Minnesota

MT..... Montana

ND..... North Dakota

NIR..... Near-Infrared Reflectance

NIV..... Nivalenol

NS..... Northern Spring

ppb..... Parts per Billion

ppm..... Parts per Million

QoIs ..... Quinone Outside Inhibitors

RS..... Red Spring

SAB ..... Shrunken and Broken Kernels

SAS ..... Statistical Analysis Software

SD..... South Dakota

SDH..... Succinate- Dehydrogenase

SIM..... Scanning Electron Microscope

SQR ..... Quinone Oxidoreductase

TCA..... Tricarboxylic Acid

TDF ..... Total Defects

TDI ..... Tolerable Daily Intake

TKW..... Thousand Kernels Weight

TWL ..... Test Weight lb/Bushel

USDA ..... United States Department of Agriculture

15-ADON ..... 15-Acetyl Deoxynivalenol

3-ADON ..... 3-Acetyl Deoxynivalenol

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INTRODUCTION

Hard Red Spring (HRS) wheat is grown in multiple U.S. states, including North Dakota, South Dakota, Minnesota, and Montana, and represents about 25% of the total U.S. wheat production. The word "hard" is an indicator of the endosperm texture. This class of wheat is sown in the spring and collected at the end of summer or during the fall. Hard Red Spring wheat is high in protein content; therefore, it is used to produce breads (Vocke & Ali, 2013).

Fusarium Head Blight (FHB), also known as scab, is caused by the fungus *Fusarium graminearum* and can attack wheat and some grains, transforming a field of green florets into discolored florets, especially after severe weather, such as rain coupled with a high level of humidity, during the flowering or grain-filling stages, which create favorable conditions for the disease (McMullen et al., 1997). The FHB fungus can produce deoxynivalenol (DON) (also known as vomitoxins), which is a type of mycotoxin that induces vomiting in humans and animals. In addition, FHB can cause yield loss; kernels become shriveled and have a low-test weight; and wheat becomes subject to a grade reduction. As a result, processing or exporting wheat to other countries is hindered (McMullen et al, 1997). Contamination with the DON toxin can influence the foreign or local market exchange (Miller et al., 2014). There is a limit of 2,000 µg/kg of DON for wheat that is intended to be used for further processing, and 1,000 µg/kg of DON for wheat derivatives such as flour, meal, semolina, and flakes (Codex Alimentarius Commission [CAC], 2019).

The *Claviceps* fungi cause a disease known as ergot. The *Claviceps* fungi contain about 40 recognized species, and 10 species are commonly found in the United States. One of the top three is *Claviceps purpurea* (*C. purpurea*), which has a great economic influence, along with *C. africana* and *C. paspali* (Alderman, 2006). *Claviceps purpurea* is a fungus that is commonly

found in rye, but it is can also infect several host crops (e.g., wheat, oat, or barley) (Friskop et al., 2018). *Claviceps purpurea* infects several hosts, having up to 400 host species, such as wheat, rye, and barley (Alderman, 2006). These fungi can produce ergot alkaloid (EAs) toxins, which, upon consumption, adversely affect the health of humans and animals (Friskop et al., 2018). Human ergotism is characterized as gangrenous, where blood flow decreases to the extremities, in addition to the convulsive ergotism that disrupts the nervous system (Bennett & Bentley, 1999). Ergot bodies are characterized by a black, purplish sclerotium that projects instead of the wheat kernel at the host's spike. Cool (60-80 F) and wet weather are favorable conditions for ergot to infect grains (Friskop et al., 2018).

In the United States, the limit for ergot sclerotia was established as the percentage of sclerotia weight per grain weight; the limit was 0.05 % for wheat (Friskop et al., 2018). The maximum level of ergot sclerotia was established as "0.05 % or 500 mg/kg weight per weight (w/w) sclerotia" in Durum and common wheat (European Food Safety Authority [EFSA], 2012). Recently, the European Union's (EU) "Commission Regulation (EU) 2021/1399 of 24 August 2021 amending regulation (EC) No 188/2006" reported the limit of EAs as 100 ppb when milling products such as wheat, barley, and oats with an ash content less than 0.9% (refined white flour), and limited the EAs level to 150 ppb for milling products with an ash content above 0.9% (whole-grain flour) (Official Journal for the European Union, 2021).

Certain parameters, such as test weight, damaged kernels, shrunken and broken kernels, foreign matter, and total defects, are required for wheat grading (Federal Grain Inspection Service-U.S. Department of Agricultural [FGIS-USDA], 2014b). The non-grading factors are moisture content, protein content, dockage, 1,000-kerneal weight, ash content, kernel size, and falling number (U.S. Wheat association [USW], 2020). Some previous studies assessed these

quality parameters in relation to FHB or DON production. No study was performed to assess these quality parameters in relation to EAs. The alteration of the grading or non-grading factors can result in safety and quality concerns, rendering the wheat unsuitable for human consumption.

Another part of this study is to assess the fungicides' effect on the level of saprophytic *Fusarium* and total EA toxins with ergot sclerotia of a confidential HRS wheat cytoplasmic male sterile (CMS-HRS wheat) line.

The Fungicide Resistance Action Committee (FRAC), is an international organization that offers guidelines about how to use fungicides and to reduce fungicide resistance (Mueller et al., 2020a). The FRAC codes are expressed in letters and numbers to identify each fungicide group, and the numbers reflect the order in which the products were first introduced to the market (FRAC, 2005). This study focuses on fungicides that contain active ingredients under the following three codes: FRAC code 3, FRAC code 7, and FRAC code 11. Carboxamides under FRAC code 7 trigger Complex II for fungal respiration (Succinate-Dehydrogenase, SDH). On the other hand, the chemical group of FRAC code 11 targets Complex III of fungal respiration, specifically ubiquinol oxidase or the (ubiquinol oxidation, Qo) site. Quinone outside Inhibitors (QoIs), such as pyraclostrobin and fluoxastrobin are also known as strobilurin. DeMethylation Inhibitors (DMIs), such as triazoles, are under FRAC code 3 target C14-demethylation, and they disrupt sterol biosynthesis in fungi (FRAC, 2005).

Fungicides are selectively toxic, and once they penetrate to the host plant, the active ingredients target the fungus' cell without being toxic to the plant (Edgington, 1981). In the late 1990s, positive research outcomes regarding the use of tebuconazole to control FHB contributed to the great acceptance of fungicides applications for this disease (McMullen et al., 2012). Demethylation inhibitors (DMIs) are labeled for U.S. use to control FHB infection and DON

production or to eliminate other diseases that are related to fungal infection. However, utilizing QoIs can pose a food-safety risk because they can increase DON level. The combination of DMI+QOI is labeled to control other wheat diseases but is not recommended to control FHB or the production of DON. It was also recommended that fungicide application be timed to control FHB during the heading stage (Feeks 10.5), at the commencement of anthesis (Feeks10.51), or 5 days after anthesis stage starts (McMullen et al., 2012).

Applying these treatments with open flowers can possibly injure the ovaries (Sheshegova & Shchekleina, 2020), and a single application of the fungicide is more cost-effective than multiple applications. On the other hand, panicle flowering is not happening at the same time, so multiple fungicide applications can protect the plant (Prom & Isakeit, 2003).

Previous research regarding the use of fungicides to control *C. purpurea* is limited, and no prior studies investigated the total EAs level after fungicides application. Kaur et al. (2018) tested the effect of the Priaxor and Quilt fungicides on honeydew production as well as the severity of *C. purpurea* in perennial ryegrass. Wu et al. (n.d.) evaluated the incidence of ergot bodies and the ascospores production of *C. purpurea* in Kentucky bluegrass that was treated with Quilt. Walenta et al. (2009) used Quilt fungicide to evaluate the percentage of ergot *C. purpurea* infection in Kentucky bluegrass. Dung et al. (2018a) used Priaxor and Quilt, along with other fungicides to evaluate the severity and incidence of *C. purpurea* in two Kentucky bluegrass varieties. Another study by Dung et al. (2018b) aimed to control the germination of ergot and ascospore production by applying fungicides to the soil rather than protecting the flowers by using an artificial infection of *C. purpurea*.

According to Lorenz and Hoseney (1979), hybrid wheat (*Triticum aestivum L. em. Thell.*) was used to increase the production of wheat; however, the northern Great Plains had suffered

from *C. purpurea* fungi in hybrid Spring wheat. The cytoplasmic male-sterile spring plant is extremely vulnerable to ergot infection because of the open floret that is waiting for pollination to occur (Lorenz & Hoseney, 1979). Ergot alkaloids are produced when the sclerotium is formed, but not during the honeydew or conidia production. The total EAs level in the individual sclerotium is between 0.01 and 0.5%, which is based on the location and weather (Lorenz & Hoseney, 1979).

The current study uses Miravis Ace (FRAC codes 3+7) because it was utilized with previous research to suppress DON content. Singh et al. (2021) used Miravis Ace to control FHB in wheat, but the application was at different Feeks (different wheat growth stage). They found that applying the fungicide before anthesis, at 50%-head emergence (Feeks 10.3), was significantly effective with reducing DON content by 69%. No prior study used Sphaerex (FRAC code 3). Quilt (FRAC codes 3+11) and Priaxor (FRAC codes 7+11) were utilized by Kaur et al. (2018) and were effective for reducing the production of honeydew (sticky liquid form during *C. purpurea* infection) and limiting the severity of *C. purpurea* in perennial ryegrass.

This study will add to what is already known about DON in relation to the grading and non-grading factors and will fill in the gap about the relationship of EAs and the wheat's quality traits. This study's goal was to evaluate the production of DON and EAs toxins for both fungi (*Fusarium sp.* and *C. purpurea*) in relation to the wheat's quality parameters for HRS wheat-survey samples obtained from four growing areas in four states (2019-2020). This study's goal was also to go a step further by evaluating the effect of four fungicides (Sphaerex, Miravis Ace, Quilt, and Priaxor) on the production of saprophytic *Fusarium* toxins and EAs in the ergot sclerotia of a confidential HRS wheat cytoplasmic male sterile (CMS-HRS wheat) line, which is

extremely susceptible to ergot infection. Natural *C. purpurea* inoculum was relied on for the field experiment. The *fusarium* toxins in the samples were results of natural saprophytic colonization on ergot sclerotia. The four fungicides were applied once before anthesis (full-head stage at Feeks 10.5).

Overall Goal

The overall goal of this research is to extend the knowledge about the presence of two predominant mycotoxins, DON of the *Fusarium* species and EAs of the *C. purpurea*, that could threaten the safety of grains intended for human consumption. HRS-wheat survey samples for two different seasons (2019 and 2020) were obtained from different growing regions located in four states (Montana, Minnesota, South Dakota, and North Dakota) and analyzed for the presence of these two toxins. Variations with the grading and non-grading factors for the HRS-wheat survey samples between the seasons and regions, and the relationship of DON and EAs with these factors were discussed.

Another part of this research evaluated the effect of four fungicides: Sphaerex, Miravis Ace, Quilt, and Priaxor, and non-treated control (single application before anthesis, full-head stage, at Feeks 10.5) on the production of saprophytic *Fusarium* toxins and EAs in ergot sclerotia of a confidential CMS-HRS wheat line. Prior to this study, there was no research that examined the content of total EAs after fungicide applications. This research presents a starting point for the impact of fungicides on the control of ergot sclerotia weight and the production of total EAs.

Hypothesis

1- We hypothesized that environmental factors (years and locations) have effects on HRS-wheat quality traits and associated toxins.

2- The use of fungicides before anthesis on CMS-HRS wheat can mitigate ergot sclerotia weight and total EAs productions.

Objectives

1- To compare and quantify the concentrations of DON and EAs in HRS-wheat survey samples (2019 and 2020) in relation to wheat's quality parameters.

2- To describe the physical and compositional characteristic of ergot sclerotia from samples with four different fungicide treatments.

3- To compare the toxins' composition of the ergot sclerotia from samples with four different fungicide treatments.

LITERATURE REVIEW

This chapter will cover background information related to wheat, wheat structure, and some parameters used to determine the wheat's quality. Moreover, this chapter includes wheat diseases, specifically FHB and ergot; health problems associated with these diseases; life cycles; and the toxicity limit for food and feed. In addition, the fungicides used to mitigate ergot's severity are discussed.

Cereal Production Trends

Cereal grains are an essential source of food for daily human meals. Recently, the annual production of cereals reached 2.3 billion tons. However, 1 billion tons of cereals are utilized for food; 750 million tons of cereals are produced for animal feed; and 500 million tons are left for other uses. The global growth rate for cereal production was reduced annually by 1%, 1.6%, and 3% in the 1990s,1980s, and 1970s, respectively. The growth rate for cereal production between 2002 and 2003 was estimated to be zero, but in the following years, the growth rate increased from zero to 2.3%. In many countries that produce cereal, the product's price has increased since 2005 due to adverse weather conditions coupled with less investment in cereals, which all contributed to an increased in price globally. However, wheat can survive in severe weather conditions, can achieve high productions level, and can be stored for a long period of times, leading to urbanization in many areas. In recent years, the production of wheat for food, animal feed, or non-food uses was approximately 65%, 17%, and 12%, respectively (Food and Agricultural Organization [FAO], 2013).

United States' Wheat Production and Standard

Wheat (*Triticum aestivum L.*) is considered to be one of the three most important crops in the United States, after corn (*Zea mays subsp*) and soybeans (*Glycine max*). Approximately

1.697 billion bushels of Spring wheat, Winter wheat, and Durum wheat were anticipated to be produced between 2021and 2022, utilizing 38.1 million acres of planted land in the United States. However, compared to 1981, wheat production and cropland declined by roughly 1.1 billion bushels and 40 million acres, respectively. Winter wheat is about 70% of the entire U.S. wheat production. Spring wheat represents 25% of the entire U.S. wheat production. Durum wheat is grown the least with approximately 2-5% of the entire U.S. wheat production. The United States is exporting wheat to other countries, and it comes after Russia and the European Union, representing 6-7% of the total wheat production globally. After 2000, the United States with other exporting countries, such as Canada, Australia, Argentina, and the former Soviet Union, continued to represent 90% of the global wheat trades (USDA-Economic Research Service [ERS], 2020).

According to the Code of Federal Regulation (CFR) § 810.2201, wheat classes consist of Durum wheat, Hard Red Spring wheat, Hard Red Winter wheat, Soft Red Winter wheat, Hard White wheat, Soft White wheat, Unclassed wheat, and Mixed wheat (USDA-FGIS, 2014b). This dissertation focuses on HRS wheat which includes Dark Northern Spring (DNS) wheat, Northern Spring (NS) wheat, and Red Spring (RS) wheat (USDA-FGIS, 2014b).

Hard Red Spring wheat can be found in many countries in the U.S. states, such as North Dakota, Montana, Minnesota, and South Dakota. The protein content for HRS wheat is high; therefore, the crop can be used for special kinds of breads or mixed with other kinds of wheat that contain less protein. In 1997, the demand for wheat was negatively affected by consumer trends, specifically the shift to consuming less carbohydrate. As a result, since 2000, there was a decreased demand for breads and flour (USDA-ERS, 2020).

Hard Red Spring (HRS)Wheat

Hard Red Spring wheat is considered to be one of the most essential wheat classes due to its high milling yield; good baking quality; and use for different kinds of bread, such as rolls, croissant, and buns. The HRS wheat's protein content is high (12.0-15.0%), and the crop contains robust gluten content and can easily absorb water (Shelton & Martin, 2008).

The estimated Spring and Durum wheat productions in North Dakota are roughly 50% of the United States' production. This amount of North Dakota wheat can produce 11.6 billion bread loaves annually. These red-color kernels are also known as DNS wheat. The seeds are best planted during Spring (April-May) and harvested when the wheat is grown and reaches 2-4 feet. Wheat requires enough water to grow, and is usually harvested during the late summer, which starts at the beginning of August, until mid-September (North Dakota State Government [NDSG], 2019). From 2003 until 2014, the estimated annual production of HRS wheat in the midwestern and northeastern portions of the United States was 15,228 and 45,970 tones, respectively.

Special Grades of Wheat

Ergoty wheat refers to the ergot sclerotia level in wheat which is above 0.05 %. Garlicky wheat refers to wheat with green garlic bulblets, roughly two or above in 1,000 g of wheat. Smutty wheat refers to smut balls or fractions of smut balls in 250 g of wheat; there can be no more than 30 smut balls (USDA-FGIS, 2014b).

Wheat Climate

Wheat can be planted in the tropics, in areas close to or far from the equator, by utilizing irrigation. During the summer season, wheat can grow in the subtropics with rainfall, but irrigation systems can be used during wintertime. Spring wheat needs approximately 100-130

days to grow. It requires less snow and cold weather (5 °C), but wheat might be negatively affected by frost. During the tillering stage, a temperature from 15°C -20 °C is ideal for growth, and a temperature of 18 °C is suitable for the ripping stage (FAO, 2021).

Wheat Structure

The major wheat structures are the hull and bran layers, the germ, and the subaleurone layer. In general, the grain structure consists of a one-seeded fruit known as the caryopsis, and the fruit's coat is combined with the kernel. When the fruits mature, the pericarp is integrated with the seed wall. The bran layer consists of the pericarp, seed coats, nucellus, and aleurone cells. The endosperm is a big portion of the kernel in order to store food, and a small portion of the kernel is for the embryo (Figure1). The seed's shape is rounded transversely, with a lengthwise crease. The stigmatic end contains small, thin hairs called the brush (Pomeranz,1982).

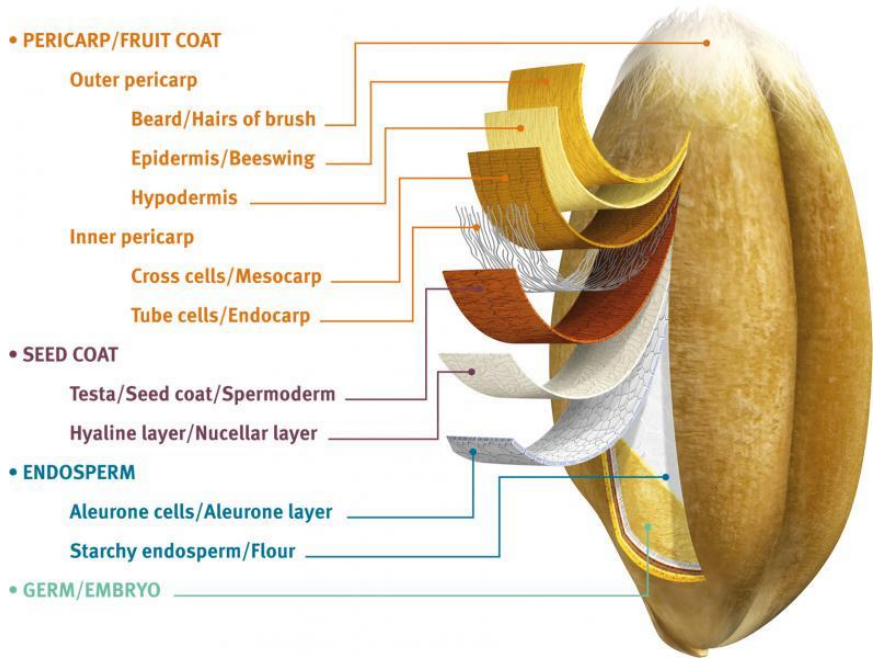

Figure 1. Wheat seed structure (Image from: GoodMills Innovation GmbH).

Bran Layer

In the bran layer, the tube cells and cross cells represent the pericarp's inner layer while the hypodermis and epidermis are the outer layers of the pericarp (Pomeranz,1982). The order of the bran layers from the outside to the inside is as follows: outer pericarp, inner pericarp, seed coat, hyaline layer, and aleurone layer (Evers & Bechtel, 1988; Rosa-Sibakov et al., 2014). The inner layer is more susceptible to molds due to its thin, walled cells and the presence of intracellular space that enables water movement. The bran layer is protected by the hull, especially in the field when utilizing mechanical harvesting or post-harvesting (Pomeranz,1982). Bran contains fiber and ash, and represents about 14% of the kernel's weight (Finnie & Atwell, 2001).

Germ Layer

The germ, containing some lipids and other nutrients, is about 3% of the kernel (Finnie & Atwell, 2001). Some of these nutrients are sugar, proteins, vitamins, and sterol (Rosa-Sibakov et al., 2014). The sugars are mainly sucrose (Pomeranz,1982). In addition, the germ has the scutellum and the embryotic axis (Rosa-Sibakov et al., 2014; Barron et al., 2007). The scutellum and embryotic axis are formed, right after the fertilized egg splits to make the embryo. Then, the embryo starts to develop within 7 days post-anthesis (Shewry et al., 2012). The embryotic axis encompasses the plumule and the radical, while the scutellum stores food (Pomeranz,1982).

The Endosperm

Flour is chiefly made of the endosperm (Lersten, 1987). The endosperm contains starch granules encircled by proteins (Pomeranz,1982). It is also surrounded by the aleurone layer (Lersten, 1987). The aleurone layer separates the bran from the endosperm (Evers & Bechtel, 1988). The proportion of the starchy endosperm is 82.7%-83.7%, and glucose compromises

about 96% of the endosperm (Barron et al., 2007). The endosperm contains a high starch and a medium protein content (Evers & Bechtel, 1988). The starch within the endosperm is occurred during the kernel-filling stage. The endosperm delivers essential nutrients to the embryo (Lersten, 1987).

Parameters Used for Determining the Wheat's Quality

According to the United States Wheat Associates (USW), there are five grading factors to help evaluate the wheat samples' physical characteristics and to determine if the samples are appropriate for milling (USW, 2020). The grading factors include test weight, damaged kernels, foreign material, shrunken and broken kernels, and total defects (FGIS-USDA, 2014b)**.** There are three subclasses of HRS wheat, and they are classified, based on the percentage of the dark, hard, and vitreous (DHV) kernels, into Dark Northern Spring (DNS), Northern Spring (NS), and Red Spring (RS) wheat (FGIS-USDA, 2014b)**.** The non-grading factors are moisture content, protein content, dockage, 1,000-kernel weight, ash content, kernel size, and falling number (USW, 2020).

Wheat-Grading Factors

Grading factors can be performed for wheat, and the results can be compared with previous wheat reports from different seasons to detect wheat-quality trends. The Federal Grain Inspection Service (FGIS) established grading factors with numerical grades (U.S. Nos.1 to 5) that are required for testing wheat after the removal of dockage (FGIS-USDA, 2014a).

Test Weight

Test weight can be determined by knowing the sample's density, using pounds per bushel (lb/bu) or kilograms per hectoliter (kg/hl). The test weight can be used as a marker to determine

if there are issues related to the wheat's growing time, harvesting, or how the samples look in general (USW, 2020).

Damaged Kernels

Damaged kernels are a factor for knowing the following: the presence of insects, possible disease, wheat-sprout problems, or freeze damage (USW, 2020). In 1993, the upper limit for damaged kernels was determined to be 2.0% for No.1 DNS wheat. Damage caused by FHB can negatively affect wheat yields, causing a discolored and shrunken "tombstone" appearance in grain, and reducing the quality in wheat (McMullen et al., 1997). Dexter et al. (1996) found that an increase for the percentage of *Fusarium*-damaged kernels corresponded with higher DON levels in Canadian-wheat cultivars. To evaluate the damaged kernels, an inspector collects 50 g from the heat-damaged sample and 15 g from all other types of damaged kernels in order to inspect the samples for color variations, or any physical and biological objects (Shelton & Martin, 2008).

Foreign Material

Foreign material refers to the matters that are left after using a Carter Dockage Tester or using hand sieves (USDA-FGIS, 2009). These foreign objects can negatively influence the milling process and the flour's quality (USW, 2020). The inspector detects any objects after removing dockage along with the shrunken and broken kernels for a 50 g sample (Shelton & Martin, 2008). Some foreign material is similar to the size of wheat kernels; therefore, it is hard to remove the foreign material with the Carter Dockage Tester (Mercier, 1989). Foreign material can be divided into organic and non-organic matter. Organic matter is derived from plants such as chaff, stalks, and weed seed. Non-organic matter is originated from objects such as stones, glasses, and metals (Awulachew, 2020).

Shrunken and Broken Kernels

Shrunken and broken kernels consist of wrinkled or broken kernels which are associated with a reduced flour yield after milling (USW, 2020). An inspector may obtain 250 g of the sample, place it in a certain-sized sieve, shakes for 30 times using a machine. Some traders in foreign countries may manually pick up the shrunken and broken kernels (Shelton & Martin, 2008). A broken kernel is mostly associated with damage that occurs during harvesting or that is caused by primary insects, such as *Sitophilus granaries.* During storage, intact kernels are less likely to be affected than broken kernels. However, secondary insects were more attracted to the infected, broken kernels compared to the mechanically broken kernels (Trematerra et al., 2000). Shrunken kernels tend to be affected by a high temperature. Tashiro and Wardlaw (1990) observed wheat behavior under a high temperature (36/31°C), at anthesis and 3 days post anthesis, and found malformed and shriveled kernels.

Total Defects

"Total defects" refers to the amount after adding up the damaged kernels, foreign materials, and shrunken and broken kernels to obtain the total defects (USW, 2020; Shelton & Martin, 2008). A representative sample may contain "shrunken and broken kernels, discolored, germinated, immature, heat damaged, and frost damaged" (Awulachew, 2020, p.21).

Dark, Hard, and Vitreous Kernels

"Vitreous kernels" means that all kernels in the HRS wheat are dark in color, with no visible starch spots or softness. This feature can be calculated by utilizing the percentage of the kernel that is picked by hand from a 15 g wheat sample (USW, 2020). Kernel vitreousness is one factor that affects the kernels' hardness because starch granules are confined by the proteins to create robust kernels (Pasha et al., 2010). Non-vitreous kernels contain spots with more starch,

while vitreous kernels are more likely to be translucent (Baasandorj et al., 2015). Moreover, Konopka et al. (2015) explained that vitreous kernels are more likely to be heavy in weight, darker in color, and more firm compared to the starchy (non-vitreous) kernels. According to the USDA-FGIS's (2016) for grain-grading primer, HRS wheat can be classified into three subclasses: DNS wheat if the wheat contains >75% DHV kernels, NS wheat if the wheat contains 25-75% DHV kernels, and RS wheat if wheat has <25% DHV kernels.(USDA-FGIS, 2016). Wang et al. (2002) found that protein and starch levels, wheat color and hardness, and light diffusion within the endosperm are factors to determine DHV versus non-DHV kernels when using NIR spectroscopy. Dexter and Edwards (1998) also indicated that vitreousness is closely related to protein content, and if the protein content is within the average, then the vitreousness can be overlooked.

Wheat's Non-Grading Factors

Wheat's non-grading factors are not required, but they can be performed at many official testing locations if there is a written agreement to do these tests (Shelton & Martin, 2008). The non-grading factors are moisture content, protein content, dockage, 1,000-kerneal weight, ash content, kernel size, and falling number (USW, 2020).

Dockage

Dockage can be removed from wheat samples by using a Carter Dockage Tester. This process helps to prepare wheat for grading (USW, 2020). Abbas et al. (1985) found that cleaning wheat with a Carter Dockage Tester was not effective for reducing the DON content, and only a slight reduction in the DON content was observed, ranging between 6 and 19% for the Winter Hard wheat samples. Dockage does not affect the wheat's numerical grading, but this parameter can be reported in other documents. Dockage can be any object that has a different size and

shape than the wheat kernels (Shelton & Martin, 2008). Dockage is measured as a percentage and is comprised of "chaff, dust, weed seeds, other grains, sand, dirt" (Mercier, 1989, p.2). Wild oats, jointed goatgrass, feral rye, cheat, and Italian rye grass are some of the most common problems associated with wheat, resulting in reduced wheat-quality parameters, such as increased dockage, a higher yield loss, and a reduced price (Fast et al., 2009). For example, dockage accounted for 82% of the total mean price discount as a result of more jointed goatgrass (Fast et al., 2009).

Moisture

The moisture content can be determined by calculating the percentage of water that is present in wheat. Moisture can be a sign of flour yield in milling. In addition, the moisture content helps millers to decide if water needs to be added to meet a specific water-level criterion in the wheat before milling. However, low moisture levels in the wheat are desired during storage. Moisture basis (mb), by means of 12%, 14%, or dry matter, is used for wheat during testing (USW, 2020). Pasha et al. (2010) mentioned that moisture content is important to determine the wheat's texture and grinding time. Also, wheat with a high moisture level is more susceptible to bacteria and fungi due to spore activation with the wheat's increased moisture content. Moreover, when storing wheat at a high moisture level, increased bacterial and fungal activity may render the wheat unfit for human consumption (Whitesides, 1995).

Protein

The protein content is the weight of kernels containing protein, expressed as a percentage. High and low protein content may play an important role when millers choose good-quality wheat (USW, 2020). Genetic, environmental, and agricultural practices all have roles in the protein content's percentage (Awulachew, 2020). Breads and pasta require a high level of

protein while other food, such as cakes or snacks, needs a low level of protein. The protein content in HRS wheat is based on the 12% standard moisture basis (mb) and can be determined by using the American Association of Cereal Chemist International's (AACCI) 39-10.01 Near Infrared (NIR) method. The flour's protein content is based on the 14% mb using the AACCI 39-10.01 NIR method (USW, 2020). A combustion nitrogen analyzer can be utilized to determine the protein content due to the analyzer's accuracy, being free of chemicals, and being time efficient (Shelton & Martin, 2008). Also, a high protein level in wheat is linked to hard kernels, robust gluten, and desired end products (Pasha et al., 2010).

Ash

Ash contains minerals that are abundant in the wheat's bran layer. The percentage of ash content in flour indicates that the flour is tainted with bran, which is present in the flour as a dark color. Therefore, white flour requires a very low percentage of ash, but a high percentage of ash is needed for whole wheat; the method used to determine the ash content is AACCI 08-01.0 (USW, 2020).

Kernel Size

The kernel weight's percentage is based on small, medium, and large kernels. Kernels with less variation in size, or with large sizes, are more desired to enhance the flour's yield for milling. The sieve sizes used to separate the kernels for HRS wheat are Tyler No.7, with an open size of 2.82mm, and Tyler No. 9, with an open size of 2.00 mm (USW, 2020).

Thousand-Kernel Weight

Thousand-kernels weight means measuring the weight of 1,000 seeds using an electronic seed counter; the seeds are weighed (in grams) to anticipate the flour yield or kernel size (USW, 2020). The thousand-kernels weight could be affected by several factors; one of them is rain. A

study by Hirano (1976) observed the mean value of 1,000- kernels weight after harvesting mature kernels at different times of the repining stages under artificial rain. Hirano (1976) found that, compared to the control, the 1,000- kernels weight decreased at 16-19 days and 23-26 days after the heading stage when exposed to the artificial rainfall. The 1,000- kernels weight started to increase at 37-40 days after heading phase.

Falling Number

Another non-grading parameter is the falling number. Hagberg (1960) developed this simple rapid method, compared to other time-consuming methods, to determine the approximate amount of α-amylase activity in grain samples. A plunger is used to measure the time needed for the plunger to fall through a tube containing a slurry of flour and water that is heated in a boiling water bath. Falling number is reported in seconds and if the falling number value is high, then the α-amylase activity is low. Conversely, if the falling number value is low, then the α-amylase activity is high (USW, 2020). Generally, a falling number value of 300 seconds or higher indicates sounds wheat with low α-amylase activity. On the other hand, a low falling number that is less than 250 seconds indicates high α-amylase activity and sprout damaged wheat (Shelton & Martin, 2008). When harvesting and milling sprouted wheat, there are issues with the end product quality (Evers & Bechtel,1988). While processing, a high level of α-amylase in the flour is not recommended because it induces poor-quality dough (sticky) for the end products. When making bread, yeast produces α-amylase to support the bread's volume, so high enzyme activity in the flour will cause excess hydrolysis of starch (USW, 2020).

Mycotoxin Diseases Related to Wheat

Between 1960 and 1975, researchers started to investigate mycotoxins, and the investigators found more than 300 mycotoxins. Some mycotoxins caused a lot of human and

animal diseases. However, mycotoxins produced by different kinds of fungal species have a small molecular weight and are composed of various chemical structures with countless biological effects. Some of the major mycotoxins are trichothecenes, aflatoxin, patulin, citrinin, zearalenone, patulin, and EAs (Bennett & Klich, 2003).

Fusarium Head Blight

Fusarium Head Blight (FHB) is a fungal disease caused by *Fusarium graminearum* (*F. graminearum*). This fungus causes infection to wheat during kernel's formation, especially during flowering stage throughout the kernel-filling stage. *Fusarium* has several species, but in North America, *F. graminearum* is mainly detected during FHB infection. In the past few years, FHB causes outbreaks in several places, such as the Unites States, Canada, Asia, Europe, and South America. Moisture and warm temperature are perfect conditions for the growth of fungus (McMullen et al., 2012). Hard Red Spring wheat and other grains are subject to fungi during production and storage (Bianchini et al., 2015)

Fusarium Mycotoxins

Several toxins can be produced by different *Fusarium* fungi in grains, and it is important to identify these toxins to better understand the fungi. *Fusarium culmorum* produces DON, 3-acetyl deoxynivalenol (3-ADON), 15-acetyl deoxynivalenol (15-ADON), nivalenol (NIV), fusarenone-X (FUX), and zearalenone (ZEN), while *F. graminearum* produces DON, 15-ADON, NIV, FUX, and ZEN. Also, *F. sporotrichioides* produces T-2 toxin, HT-2 toxin, neosolaniol (ENNS), diacetoxyscirpinol (DAS), FUX, and ZEN. Finally, *F. poae* produces T-2 toxin, HT-2 toxin, NIV, DAS, and FUX, *F. verticilloides* produces Fumonisins (Magan et al., 2002).

Trichothecenes

The name Trichothecene is obtained from Trichothecin, which is known to be the first family member. Trichothecenes are produced by different fungi, such as *Fusarium, Myrothecium*, *Phomopsis*, *Stachybotrys*, *Trichoderma*, and *Trichothecium* (Bennett & Klich, 2003). Trichothecenes are classified into four group types: A, B, C, and D. The type A and B toxin groups are commonly known for their relationship with human and animal food (Habler et al., 2016). Trichothecenes are also divided into macrocyclic or nonmacrocyclic. The nonmacrocyclic type contains two common groups, A and B. Group A contains a hydrogen or ester located at C-8, group A includes T-2 toxin, ENNS, DAS (Bennett & Klich, 2003), and HT-2 toxin (Kushiro, 2008). Group B has a ketone that includes FUX, NIV, DON (Bennett & Klich, 2003), 3-ADON, and 15-ADON (Alexander et al., 2011). Grains, including barley, oats, rye, and wheat, are subject to pathogens that are synthesized by *F. graminearum*. The amount of toxins produced by all types varies, depending on the fungus growth, weather, area, and year. Deoxynivalenol is one of the most abundant mycotoxins that is found in grains (Bennett & Klich, 2003).

Zearalenone

Zearalenone (ZE) is another *Fusarium* mycotoxins. The old name for the ZEN was F-2 toxin. This mycotoxin's chemical structure is "resorcyclic acid lactone [6-(10-hydroxy-6-oxo-*trans*-1-undecenyl)-β-resorcyclic acid lactone ($C_{18}H_{22}O_5$, MW: 318.36, CAS 17924–92-4)]" (Ji et al., 2019, p.5). Around 16 countries have established a limit, ranging from 50 to 1,000 μg/kg, for the estrogenic zearalenone mycotoxin in cereals and maize (FAO, n.d.).

Fusarium Head Blight's Life Cycle

Fusarium Head Blight is also known as scab. Disease-causing species in wheat include *F. graminearum, F. avenaceum, F. culmorum and F. poae.* These fungi can also cause an FHB infection in other hosts, such as barley and oats. During the asexual phase, *F. graminearum* invades what remains from the crop after harvesting, such as wheat straw, stalks, and overwinters. The asexual phase in the *F. graminearum* involves the production of macroconidia, also known as asexual spores, from the phialides (sporodochia), which can be spread to other plants by winds or rain. Favorable conditions, such as warm and damp weather, can trigger the sexual stage where the fungus (*Gibberella zeae*) grows on wheat residues. The *Gibberella zeae* fungus is a fruiting body (perithecium), and it produces ascospores, or sexual spores, in the air (Schmale III & Bergstrom, 2003).

The most vulnerable wheat varieties might pick up the macroconidia and ascospores, which can cause an infection, especially during the flowering phase. The fungus might penetrate during early anthesis, which prevents the floret from growth and the kernels from emergence. If the infection starts during late anthesis, the kernels appear deformed, shrunken, and broken. In contrast, if the infection occurs after the kernels are emerged, the kernels will appear normal but tainted with DON (Figure 2; Schmale III & Bergstrom, 2003).

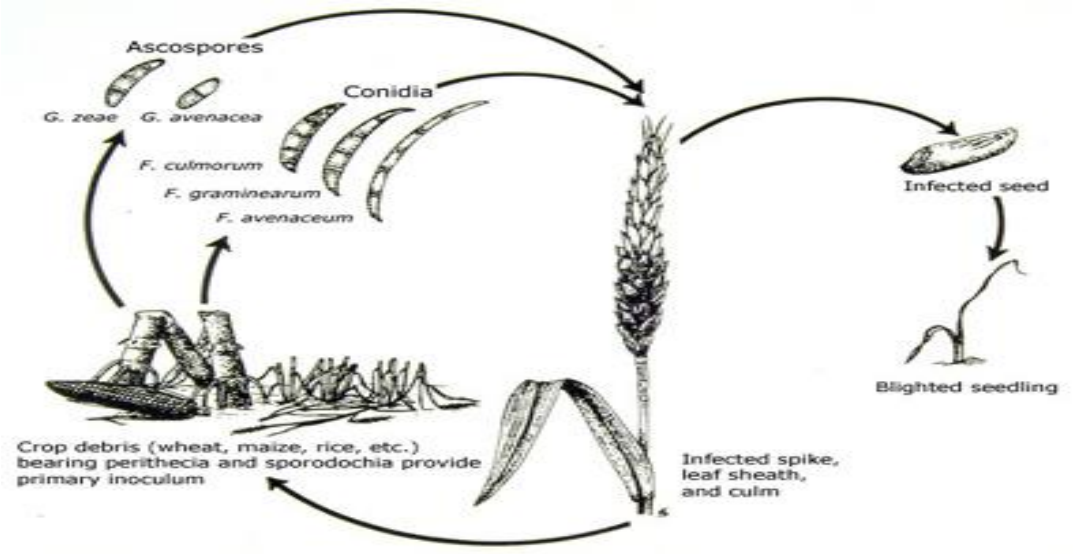

Figure 2. FHB life cycle. Reproduced, by permission, from Schmale III, D. G. and Bergstrom, G. C. 2003. Fusarium Head Blight in wheat. *The Plant Health Instructor*. DOI:10.1094/PHI-I-2003-0612-01.*Updated 2010*.

Human Health

Mycotoxicosis can be divided into acute and chronic conditions. Patients with acute-toxicity can develop symptoms immediately after exposure to the mycotoxins. On the other hand, patients with chronic toxicity, who have been exposed to a low concentration of mycotoxins for a long time, can develop cancer, kidney problems, and weak immune systems (Bennett & Klich, 2003). Researchers explain how some groups in a population are more susceptible to mycotoxins. For instance, Hispanic people are at a higher risk from mycotoxins due to their large consumption of corn-based foods compared to Americans (Barrett, 2000).

Other factors that influence the mycotoxin's effect on people's health are age, sex, weight, diet, the amount of toxins to which they are exposed, and the co-occurrence of other mycotoxins. For example, a young child might be more severely affected when exposed to mycotoxins than an adult. The chronic conditions from mycotoxins can also be divided into three groups: mutagenic, carcinogenic, or teratogenic. Aflatoxins are known to be human carcinogens, but trichothecenes and ZEN are not. Another factor that increases the exposure to mycotoxins is

related to the poverty level. People tend to consume what is available for them, even if poor methods were used to prepare the food during any stage of the food chain. Poor food handling is another issue, especially in low income countries, because there are no regulatory guidelines, which increases the risk of mycotoxin exposure (Omotayo et al., 2019).

Deoxynivalenol induces vomiting and is known as vomitoxin. The first cases occurred in Japan in 1972, after men consumed barley that was tainted with *Fusarium* fungi. When consumers ingest food that is contaminated with DON, emetic effects occur because DON moves to the brain and affects the dopamine receptors (Sobrova et al., 2010).The name vomitoxin was first proposed by Vesonder et al. (1973), after several cases of emesis occurred in swine due to the consumption of contaminated corn with multiple *Fusarium* species. Deoxynivalenol can be present in food or cereals, such as wheat, barley, oats, rye, rice, and corn. Food contaminated with DON can cause vomitoxin, which leads to vomiting, nausea, anorexia, diarrhea, and stomachaches (Yazar & Omurtag, 2008).

Outbreaks of Deoxynivalenol

In the early 1970s, further investigation of barley infected with *F.graminearum* revealed the presence of a new trichothecene (Yoshizawa & Morooka, 1973). Deoxynivalenol was associated with several outbreaks in the past. For example, in 1978, an outbreak was reported in Kashmir Valley, India. People reported gastrointestinal symptoms after consuming bread that was made with rain-damaged, moldy wheat. The tested samples were tainted with trichothecene mycotoxins, especially DON (Bhat et al., 1989). In China, several outbreaks were associated with wheat. In 1985, a number of patients reported upset gastrointestinal tract and nervous-system problems after consuming wheat infected with FHB. After two hours, the affected individuals were normal. After this issue, DON received a lot of attention as a major concern

for human health in the Henan Province. In 1991, three Chinese provinces experienced an extreme flood. After that flood, a number of people consumed moldy wheat, which caused illnesses. Also, another outbreak occurred in the same year, affecting 130,000 patients who reported gastrointestinal symptoms due to an elevated DON level in food. In 2003, approximately 5,043 people consumed food derived from moldy wheat, but only 701 patients were ill. The majority of the victims experienced nausea, acid reflux, and throat numbness, and these episodes lasted for three hours (Qiu et al., 2019).

Deoxynivalenol Levels of Concern

In order to protect humans from excessive exposure to DON, the Codex Alimentarius Commission (CAC) limited the amount of DON in food by setting specific standards. For example, 2,000 μg/kg of DON was specified for wheat that is intended to be used for further processing. Wheat derivatives, such as flour, meal, semolina, and flakes, should not go beyond 1,000 μg/kg. Also, the maximum DON level for cereal-based food that is given to infants or children was limited to 200 μg/kg (CAC, 2019). The Food Drug Administration (FDA) updated the DON advisory level as 1ppm for finished wheat products that are intended for human consumption (National Grain and Feed Association [NGFA], 2011).

In 2002, the Scientific Committee for Food (SCF) controlled DON by limiting the tolerable daily intake (TDI) to 1 μg/kg body weight (b.w.) per day. The Joint FAO/World Health Organization (WHO) Expert Committee on Food Additives (JECFA) included 3-ADON and 15-ADON, and set a group acute reference dose (ARfD) at 8 μg/kg b.w. per day (JECFA, 2010).

Fusarium Head Blight Management

Mycotoxins contamination can impact the food industry. When there is a food recall, a company should destroy the contaminated and the non-contaminated foods, which has hug costs.

In addition, this defect can influence people's confidence, and they tend to be discouraged from purchasing the affected company's products. Suppliers are then considered as untrustworthy when it comes to grain quality. One of the suggestions to reduce mycotoxins before harvesting is insect control. Insects can injure the plant and can cause the accumulation of mycotoxins. Fungicide application is another technique that is utilized to reduce contamination and to decrease the yield loss caused by FHB disease in wheat. The time and the amount of fungicide are important when there is a possible plant infection. Fungicides, such as triazoles, are used to control mycotoxins but might be susceptible to fungal resistance. After harvesting, mycotoxins are highly susceptible to contamination during storage. Forecasting models for mycotoxins can diminish this problem by predicting toxins during storage or pre-harvesting. A feed additive based on regulatory guidelines is another approach to decrease the toxins in grains. In North America, food additives need regulatory approval and more research prior to their use in order to avoid harming food such as meat, milk, and eggs (Miller et al., 2014).

Effect of the Milling Process on Deoxynivalenol and its Derivatives

Before milling, a cleaning process that removes foreign objects, such as dust, straw, chaff, and weeds, can reduce mycotoxins (Schaarschmidt & Fauhl-Hassek, 2018). While milling, the kernels are diminished to small sizes and fractions. Before dry milling, the endosperm can be softened, and the bran can be removed. With wet milling, kernels can be placed in a solution or water in order to disperse the kernel fractions to cause the endosperm to discharge starch particles (Schaarschmidt & Fauhl-Hassek, 2018).Young et al. (1984) cleaned and milled naturally contaminated HRS wheat into bran, shorts, break flour, and reduction flour. The researchers found a low level of DON in the reduction flour, and the concentration changes for the two samples were very slight (-15%, and -24% reduction). The DON concentration increased

with the other fractions (Young et al., 1984). Abbas et al. (1985) found that milling was ineffective for reducing DON concentrations, and the DON levels in the milling fractions were different. The bran had a high concentration of DON in all the Hard-Red Winter samples, and followed by shorts, reduction flour, and break flour. Also, cleaning the wheat reduced the DON concentrations slightly, but it was not effective for destroying the DON in all samples.

History of Ergot

The agricultural practice started in the Fertile Crescent region during the 9000 BC. This led to the production of good-yielding grains such as wheat (*Triticum spp*.) and rye (*Secale cereal*). As a result, Assyria and Babylonia in ancient Mesopotamia were evolved due to this dominant food supply. Nevertheless, during a rainy season, *C. purpurea* contaminates the rye's spike and produces ergot. This ergot might have been first described in 600 BC by an ancient Assyrian as "noxious pustule in the ear of grain" (Barger,1931; Van Dongen & De Groot, 1995, p.109). In the history of Rome, Lucretius (98-55 BC) described ergotism as erysipelas , but ergotism was known during the Middle Ages as "Ignis sacer", or holy fire (Barger,1931, p.43). The name "ergot" comes from the word "argot", which is a French name that refers to the cock's spur (Van Dongen & De Groot, 1995). In Germany, ergot is known as "Mutterkorn" (Mutter-mother or Korn-grain), and it is considered to be a child of the grain. In Latin, ergot is referred to as "articulum", meaning joint, which is derived from the old French description of sclerotia as a cock's spur or the word "argot" (Lee, 2009).

The incidence of ergotism appeared repeatedly throughout the Middle Ages, and rye bread was the culprit due to contamination with *C. purpurea*. Consuming contaminated rye bread caused people to have a loss of limb sensation and a disfunction of the central nervous system (Van Dongen & De Groot, 1995). Poverty and famine caused poor people to keep the spurs in

the rye. If the black spurs are not separated from the grain, the prepared bread can be contaminated, giving rise to ergotism cases. In addition, Russians were inclined to keep the spurs because the Russians believed that the spurs were essential to enhance the bread's quality (Van Dongen & De Groot,1995).

In Germany, mythologists illustrated how, during the foggy season, ergot appeared abruptly and attacked the mother grain in the field. Two forms of ergotism symptoms were described. Gangrene ergotism, which causes lost limbs and death, was first described in the *Annales of Xantenses* in Germany in 857 AD. Convulsive ergotism was first mentioned in 945 AD in Paris, France, and was described as a burning sensation in the extremities, vomiting, and diarrhea, followed by catalepsy, and death. In the Aquitaine region of southwestern France, the majority of the affected individuals (about 40,000 individuals) died. Some individuals sought sanctuary in churches, where the food was not tainted. In Vienne, France, a treatment for ergotism during this period was holy water and wine scattered over the bones of St. Antony of Egypt at the St. Antony hospitals. Afterward, these hospitals spread to around 390 regions of Europe because of their distinctive offers for non-tainted bread and their proper treatment for patients. Different names, such as St. Antony's fire, Holy Fire, or Ignis Sacer were given to ergotism because of the burning pain in the affected people's extremities (Van Dongen & De Groot, 1995).

Cultivating rye was not common in Britain because rye was considered to be a food that was consumed by poor people. However, on the European continent, growing rye was common, and rye extended from Holland to Russia and way to the eastern part of the Ural Mountains. Some of the rye was cultivated, and some of it was used as a green manure crop. Ergot (*C. purpurea*) was explained in Adam Lonitzer's 1582 book, *Kräuterbuch,* where he mentioned the

color, shape, smell, and location of the ergot body in the grains' panicle; the therapeutic use; and the dose of ergot needed for treatments. In 1658, Gaspard Bauhin was the first person to illustrate *C. purpurea*, a drawing which appeared in his *Theatrum Botanicum*. The first English description appeared in *Catalogus Plantarum Angliae* by John Ray in 1677. The word "ergot" was utilized in a publication for the first time in 1683 (Lee, 2009).

During the 17th century, 19 women in Salem, Massachusetts, were accused and executed for allegedly practicing witchcraft (Caporael, 1976). These women were accused of afflicting children with abnormal behaviors, such as speech disorders, seizures, and muscle contractions, and were convicted based on "spectral evidence" and "test of touch" (Caporael, 1976, p.21). There were some arguments about the cause of the children's convulsive symptoms during the Salem crisis. Caporael (1976) suggested that the afflicted children might have had convulsive ergot poisoning. Caporael's hypothesis was rejected by Spanos and Gottlieb (1976), who argued that the afflicted children's symptoms were not caused by contaminated grains. Spanos and Gottlieb (1976) claimed that towns other than Salem accused women of witchcraft, and none of the other towns reported the spread of ergotism. In addition, the Salem crisis was similar to what happened in Germany between the16th and 17th centuries. The authors also suggested that the afflicted girls' symptoms reflected a phenomenon where young girls claimed to be possessed by demons, which was a common occurrence at the time (Spanos & Gottlieb,1976).

Matossian (1982) agreed with Caporael's suggestion about the link between the afflicted people at the 1692 Salem Witch Trials and ergot *C. purpurea* causing convulsive symptoms after consuming contaminated rye bread (Matossian, 1982). However, Matossian published a book called *Poisons of the Past: Moulds, Epidemics and History*. Matossian stepped a little further and blamed ergot for multiple historical events, including witch persecution, a fertility decline, and

increased mortality rates among European infants before 1750. She believed that mycotoxin poisoning was responsible for the previous demographic changes, ignoring other factors, such as famine and infectious disease (plague and streptococcus). The lack of epidemiological evidence, the uncertainty, and her assertion without enough proof that attributed ergot poisoning and mycotoxins alone for the whole historical event made her beliefs subject to rejection and criticism (Hunt, 1992; Lindemann 1991; and Wilkinson,1990).

Before 1700, there was not much description about this organism's infection. Claude Joseph Geoffroy and Otto von Münchhausen in 1711 and 1764, respectively, elucidated that this organism is a fungus. In 1791, Erasmus Darwin mentioned ergot in his poem, "The Botanic Garden," and he explained to his readers in a note that ergot was a disease and that it was common in France but scarce in the United Kingdom (UK). Improved germination methods and the invention of the microscope in the 19th century helped to understand the fungus' nature. In 1850, ergot' s complete life cycle was explained by Louis René Tulasne (Lee, 2009).

Ergot's Life Cycle and Biology

Campbell (1958) described the pathway of *C. purpurea* infection in barley and stressed that the infection starts from the ovary and goes to the top. Campbell observed the infection's progression by inoculating barley through the conidial suspension of *C. purpurea* by using an atomizer at the flowering stage. During a day of inoculation, the conidia start to germinate with the slight production of mycelium. The mycelium moves close to the ovary's base. On the second and third days, the mycelium moves to the protective part of the ovary, the integument, and continues to grow vertically in the ovary to encircle the ovule. The production of conidia initiates as soon as the hypha starts to emerge at the top of the ovary. At this stage, the integument will no longer be able to resist the hypha, and the fungus colonizes the ovary

(Campbell, 1958). Luttrell (1977) suggested that conidial germ tubes in *C. paspali* make their way to the stigma and germinate on the style. Some conidial germ tubes can pass through cells to reach the style, where they proliferate and continue to the ovary. Some tubes may grow far-off from the ovary (Luttrell, 1977). Researchers were in conflict regarding ergot's the pathway during the initiation of an infection, but the clearest description of *C. purpurea*'s life cycle was explained by Tulasne in 1853 (Luttrell, 1980). Some researchers believed the infection starts in the ovary, as explained by Campbell (1958) and Luttrell (1980). Luttrell also investigated the infection pathway for *C. purpurea* and found that it is similar to *C. paspali,* although they have different structures, and the infection starts from the stigma, moving to the style and finally to the ovary. Luttrell indicated that his work is reconciled with Tulasne (1853) and others (Luttrell, 1980).

Mitchell and Cooke (1968) added the hard ergot mass contains a white medulla that holds cells in addition to a dark, pigmented part called the cortex. After the sclerotia fall on the soil, they might be partially or totally buried and may become dormant during the fall and winter. The period from fall to spring is enough time to expose the sclerotia to the cold environment which is needed to initiate gemination. However, severe cold weather may decrease the ability of the ergot mass to geminate. A favorable temperature for the sclerotia to germinate is 0 °C-10 °C; for the stroma production, the temperature is between 10 °C and 25 °C; and for the mycelium to grow, the temperature is approximately 25 °C-30 °C (Mitchell & Cooke, 1968).

Gilles et al. (1972) explained that, when the grain reaches a complete growth, ergot bodies start to drop onto the ground where the soil holds them during the winter. In the spring, ergot starts to be shaped into a small mushroom and produces ascospores. During the flowering time, the wind or an insect will carry out the spores, impacting the grain's flower. Once the seeds

start to form, the fungus begins to attack the seeds and launches into the ovaries, displaces the seeds, and forms a slimy structure called conidia or "honeydew." When the ergots mature, they start to fall on the ground, and the life cycle is repeated annually (Gilles et al., 1972).

Tenberge (1999) explained that the life cycle of *C. purpurea*'s life cycle begins with the ascospores. During the spring, ascospores may fall on the host because of the wind. Colonization may occur when the hyphae attack the ovary and grow down to the rachilla part of the ovary. However, no more parts are attacked by the fungi. In the ovary, the sphacelial stroma grows a great amount and produces spores that are eventually discharged as a syrup. The honeydew contains conidiospores that can taint plants during the flowering stage via rain splashes, insects, or from the head of one flower to the other (Tenberge, 1999). The organism can reproduce asexually by producing honeydew along with conidia during the flowering stage, which is also known as a secondary inoculum to spread the infection as much as possible in the field. It is possible that the fungi can be used as a safeguard to defend the plant and themselves from animals through the production of alkaloids (Parbery,1995, p.493).

The sexual reproduction of the fungi occurs when the male and female cells are united to produce diploid nuclei and undergo cell divisions to be in a haploid state. This process creates perithecia within the stroma that encompasses many asci. The asci include eight ascospores, where the ascospores expelled in the field by air or insects (Figure 3; Schumann & Uppala, 2000)

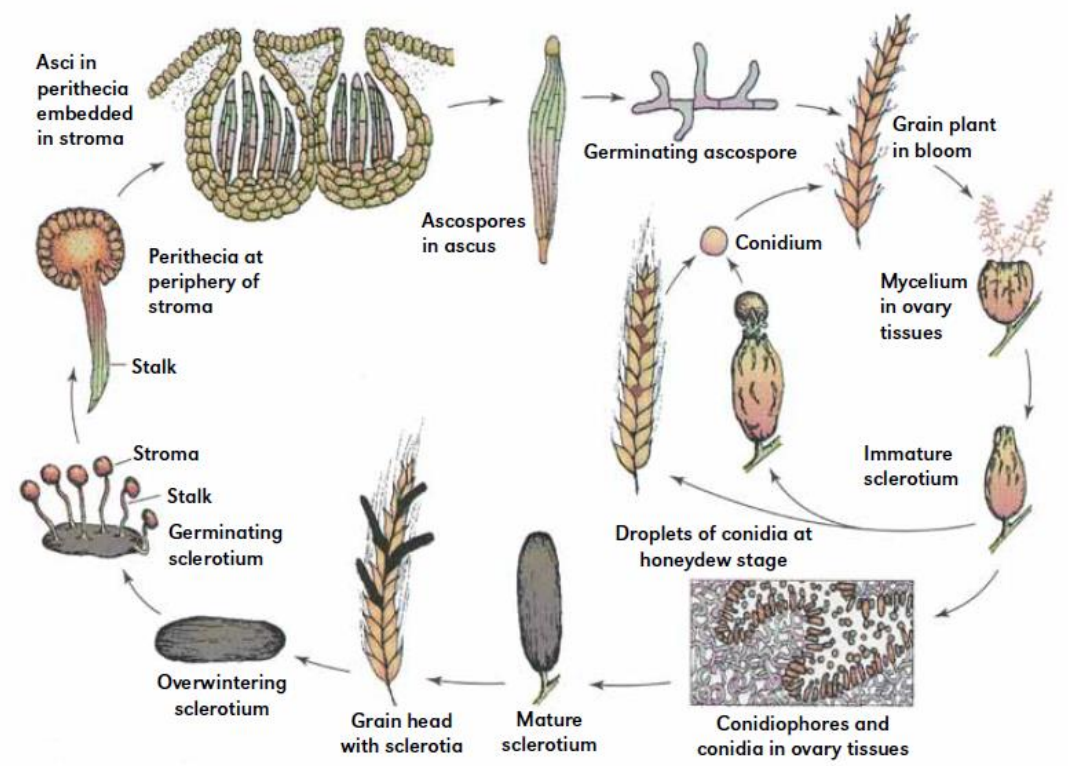

Figure 3. Ergot's life cycle. Reproduced, by permission, from Schumann, G.L. and Uppala, S. 2000. Ergot of rye. *The Plant Health Instructor*. DOI: 10.1094/PHI-I-2000-1016-01 *Updated 2017.*

The primary inoculum occurs when the ascospores are trapped by the host's stigma, germinate, and start an infection in the ovary. With asexual reproduction, the hyphae penetrate the ovary, and continue to grow until it mature, at this stage it known as the mycelium. The secondary inoculum involves the production of asexual conidia from the honeydew, which attracts insects that then spread the conidia from the infected flowers to new flowers. The production of honeydew and conidia terminates at the same time that the ovary is eaten by the hyphae. With time, the hyphae will produce a mass of tight tissue, known as pseudoparenchyma, that becomes an ergot body (Schumann & Uppala, 2000).

Effect of the Environment on Ergot

Warm weather can affect the level of the ergot infection. Kodisch et al. (2020b) reported that the low severity of ergot infection in Poland, when compared to other countries, might be

related to the warm and dry weather for a number of inculcated winter-rye samples with *C. purpurea*. Menzies et al. (2017) isolated 41 single spores from *C.purpurea* that were collected from different locations in the UK and Canada. The isolated spores were embedded in different wheat varieties. The authors found that honeydew production and the sclerotia's weight rate were higher in isolates of *C. purpurea* which were collected from the UK compared to Alberta, which implies that location plays an important role with the ergot's severity (Menzies et al., 2017).

Hybrid wheat, especially male, sterile-line-wheat, is more prone to ergot infection due to open-pollination (Watkins & Prentice, 1997). Compared to other grains' self-pollinated flower, male, sterile hybrid seed is less immune to ergot disease because the ovary is not self-fertilized. In 1997, there was an outbreak of ergot with hybrid seed in the Texas panhandle. As a result, companies tried to apply different methods and spent a lot of money to control the disease in sorghum, but to no avail. However, the outbreak became less intense after three years. The cause for the disease-intensity reduction was not determined, but some of the predictions were related to the weather conditions in 1997, which favored the growth of ergot compared to subsequent years, or more pollen management by the growers (Workneh & Rush, 2002).

Workneh and Rush (2002) investigated the reasons behind the outbreak and suggested that 1997's cool and wet weather was a factor for the ergot infection. They concluded that precipitation alone had a lesser effect for spreading ergot compared to temperature, but the precipitation did cause a higher relative humidity and a drop in the maximum temperature, which favored the growth of ergot. Montes et al. (2009) also investigated the weather's effect on the severity of ergot in hybrid and male, sterile sorghum inoculated with *C. africana*. They found that moisture can trigger ergot growth. Light rain can cause humidity that enhances *C. africana* infection, and the ideal temperature for ergot growth was 23°C for the male-sterile line. At the

same time, they found that ergot could grow with a temperature up to 38°C. Relative humidity that is higher than 30% increased the ergot's severity at the time of anthesis (Montes et al., 2009).

Chemical Structure of the Ergot Alkaloids

Ergot alkaloids (EAs), or ergolines, are secondary metabolites, meaning that they are not essential for the fungus's life development, but the contamination of EAs may have a negative effect on other organisms. Ergotamine was first extracted from the sclerotia of the genus *Claviceps* by Stoll in 1952; this procedure was done for pharmaceutical treatment purposes. A high level of EAs are found in the *Claviceps* fungi. One of the main EAs structure is the methylation of the ergoline ring on nitrogen N-6. The majority of EAs contain a double bond at two locations: C-8 and C-9 or C-9 and C-10. There are two chiral carbons in ergolene derivatives at C-5 and C-10, or at C-5 and C-8. Additionally, C-5 contains a hydrogen atom in a-β form while C-10 is always in an α-form. Ergot alkaloids can be divided into four classes based on the R substituent at position C-8 in the ergolene ring: clavine alkaloids, simple lysergic-acid derivatives, peptide alkaloids-cyclol ergot alkaloids (CEA), and lactam ergot alkaloids (LEA; Fehr et al.,1966; Flieger et al.,1997; Schreier,1958).

The lysergic-acid derivatives are identified as lysergic-acid amide, and the amid part contains peptide or alkylamide. The lysergic-acid derivatives are ergonovine (ergometrine), lysergic acid, lysergic acid α-hydroxyethylamide, ergine, and paspalic acid (Floss, 1976). Peptide ergot alkaloids consist of a lysergic-acid fragment and a tripeptide. The two major types of ergot peptide are the ergopeptine (cyclopeptide) and the lactam (ergopeptams) groups of ergot alkaloids. Both types can be classified based on the R^1 and R^2 substituents located in the ergopeptines and ergopeptams' peptide fragment. The classification for peptide ergot alkaloids

includes several groups. One of the groups is peptide ergotamine, and it includes ergovaline, α- and β-ergosine, ergotamine, and ergobine. The ergoxin group contains ergonine, α- and β-ergoptine, and ergostine. Some members of the ergotoxine are ergocornine, α- and β-ergocryptine, and ergocristine. The ergotoxam group consists of ergocornam, α- and β ergocryptam, and ergocristam. Finally, the β-ergoannam group includes α- and β-ergoannam (Komarova & Tolkachev, 2001). Clavine alkaloids is a type of alkaloids that can be found in many fungus strains, which is identified by a tetracyclic ergoline ring. Clavine alkaloids are comprised of agroclavine, elymoclavine, and festuclavine (Wallwey &Li, 2011).

Signs and Symptoms of Ergotism in Humans

Gabbai et al. (1951) explained the ergotism symptoms that occurred during an outbreak of ergot that was associated with eating rye bread at Pont St. Esprit. The authors observed how the symptoms of ergot poisoning occurred in patients for 15 days. The first symptoms started between 6 and 48 hours and were expressed as depression; agitation; nausea; abdominal pain; and, in a few cases, vomiting and diarrhea. Patients then suffered from warm and cold waves, hypersalivation, bradycardia, difficulty sleeping, stomachaches, heartburn, and dizziness. After six days, some patients had severe symptoms of vasomotor activity, cold and hot limbs, a loss of consciousness, hypertension, Babniski's sign, logorrhea, evening hallucinations, delirium, muscle cramps, hematuria, and proteinuria.

Medicinal Properties of Ergot Alkaloids

Ergot has been used as a medicine since at least 1582. Lonicer and Kreuterbuch explained that administering three ergot spurs orally can help women to increase parturition during childbirth. This dose, which is equivalent to 0.5 mg of ergometrine, was administered for many years (Moir, 1974). In 1688, Camerarius mentioned that ergot was used to accelerate labor

in some areas of Germany. In 1774, Parmentier described the use of ergot powder during labor. Ergot was first introduced to obstetrics medicine by Dr. John Stearns in the 19th century (Thoms, 1931). Stearns wrote a letter about the method of using ergot during prolonged and painful parturition: "In compliance with your request I herewith transmit you a sample of the pulvis parturiens, which I have been in the habit of using for several years, with the most complete success. It expediates lingering parturition, and saves to the accoucheur a considerable portion of time, without producing any bad effects on the patient" (Thoms, 1931, p.420).

In 1935, the name ergometrine was proposed by Dudley and Moir. They wrote, "We are now able to prove correctness by reporting the isolation of the substances to which ergot rightly owes its long-established reputation as the '*pulvis parturiens*'" (Dudley & Moir,1935, p.520). After 1935, ergometrine (ergonovine in the United States) was used as a treatment for postpartum hemorrhaging (De Costa, 2002). Although ergometrine has several medicinal uses, there are some side effects. According to Baillie's (1963) study of 266 pregnant women who needed general anesthesia for delivery and who were administered 0.5 mg of ergometrine intravenously after delivery, many women with hypertensive toxemia experienced an increased in systolic and diastolic pressure of 20 mm. Hg. However, patients with normal or chronically high blood pressure had no remarkable increase as a response to the ergometrine (Baillie,1963). Moir and Amoa (1979) compared the efficacy between ergometrine (0.5 mg) and oxytocin (10 u), which were given intravenously to control blood loss, for 88 women who had a normal vertex delivery. The study found that oxytocin was as efficient as ergometrine to impede the third-phase hemorrhage during delivery and had less ability to induce an emetic response (Moir & Amoa, 1979).

Toxicity and Human Exposure to Ergot Alkaloids

Ergot alkaloids can interact with the brain's neurotransmitter receptors and can lead to acute or adverse effects over a longer time. Therefore, the ARfD and a TDI were determined for EAs by the European Food Safety Authority (EFSA) on Contaminants in the Food Chain (CONTAM Panel). The ARfD was determined as 1 μg/kg body weight (b.w.), and the TDI was established as 0.6 μg/kg b.w. per day for the sum of the EAs. Data about human exposure to EAs were limited and were mostly for processed food. However, the data did show that the chronic dietary exposure ranged from 0.007 to 0.078 μg/kg b.w. per day for the average adult, and the acute dietary exposure was between 0.021 and 0.23 μg/kg b.w. each day for the average adult. Chronic exposure value was between 0.03 and 0.17 μg/kg b.w. per day for the average toddler, and the exposure value ranged from 0.02 to 0.17 μg/kg b.w. for the average child. High, acute dietary exposure to alkaloids among toddlers was between 0.08 and 0.42 μg/kg b.w. each day, and the exposure value was between 0.05 and 0.36 μg/kg among other children (EFSA, 2012).

Limits of Ergot Sclerotia and Ergot Alkaloids

The scientific Panel on Contaminants in Food Chains stipulated that it is difficult to establish an association between the ergot sclerotia's mass and the total EAs levels (ergoline; EFSA, 2005). This conclusion was supported study by Babič et al. (2020). They found a weak, positive correlation between ergot sclerotia and the total of EAs concentration, Babič et al concluded that an increase for the ergot mass is not an indicator of a higher EAs level. The study suggested that EAs should be monitored in cereal by using visual or chemical methods. The researchers also highlighted the importance of setting regulations for the maximum EAs level in food and feed (Babič et al., 2020).

In the United States and Canada, the maximum allowable level of ergot was established as 300 mg ergot/kg for grains (Agriopoulou, 2021; Guo et al., 2016). For cereals, the upper limit of ergot sclerotia was determined as 0.05% (500 mg/kg w/w) in durum and common wheat. However, the maximum EAs concentration was not determined. In Europe, the Directive 2002/32/EC established 1,000 mg/kg as the upper limit for rye ergot (*C. purpurea*) in feed that consists of unground cereals. Australia and New Zealand set an upper limit of 500 mg/kg of ergot sclerotia in cereals, while, in Canada, the amount of ergot sclerotia was associated with grading the grains. Therefore, the amount of ergot sclerotia was established as 0.01% and 0.1% for high- and low-quality grains, respectively. In Uruguay, a limit of 450 μg/kg was determined for EAs in animal feed, and the presence of EAs content in pigs and female rabbits feed should be avoided (EFSA, 2012). The European Union is expected to lower the ergot sclerotia percentage for unprocessed rye which is intended for human use starting July 1, 2023; the new level will be 0.02% by weight. In addition, the level of EAs present in unprocessed rye will be reduced from 500 μg/kg to 250 μg/kg, and to 20 μg/kg for infants and children. This limit will be for the total of the six major EAs and their respective epimers: ergometrine, ergometrinine; ergosine, ergosinine; ergotamine, ergotaminine; ergocornine, ergocorninine; α -ergocryptine, α-ergocryptinine; ergocrystine, and ergocrystinine (Kodisch et al., 2020 a; Miedaner et al., 2021; Raditsching, 2020). For other grains, the European commission has recently limited the EAs level to 100 ppb for milling product such as wheat, barley, and oats with an ash content less than 0.9% (refined-white flour), and the EU has created EAs level of 150 ppb for milling products with an ash content above 0.9% (whole-grain flour) “Commission Regulation (EU) 2021/1399 of 24 August 2021” (Official Journal for the European Union, 2021).

Studies Related to the Ergot-Alkaloid Content in Grains

Tittlemier et al. (2015) detected the amount of EAs in harvested samples comprised of 32 samples of Canada Western Amber Durum (CWAD), and 42 samples of Canada Western Red Spring (CWRS) wheat. In addition to the harvest samples, shipment items were comprised of 117 wheat, 46 Durum, 25 barley, and one rye sample. For EAs analysis, an internal standard, Dihydroergotamine, was utilized for the analytical process, in addition to the ultrahigh-pressure liquid chromatographic−tandem mass spectrometric (UPLC-MS/MS). The mean percentage values for ergot sclerotia were 0.030 % for CWAD compared to 0.026% for CWRS wheat that was harvested in 2011, which were both lower than the previous year. Also, between 2002 and 2013, there was an increase for the ergot infection in CWAD and CWRS wheat. In the shipment samples, wheat and Durum contained a high amount of ergocristinine (0.39 mg/kg) compared to the ergocristinine (0.23 mg/kg) for the 2011 harvest samples (CWAD and CWRS).

In general, high fractions of *R* epimers existed in the harvest sample because the samples were newly harvested. The shipment samples were stored for a long time, which catalyst epimerization causing a change of the *R* epimer into their respective *S* epimers. Another important aspect of the research was to compare the relationship between EAs and sclerotia. The study found a strong association between the ergot sclerotia's percentage and the EAs level for both the CWAD and CWRS wheat samples. The greater the percentage of ergot sclerotia, the greater the total EAs levels were in the samples, but CWAD was significantly affected. The research also found that harvest samples contained greater total EAs than the shipment samples. Among the shipment samples, the mean value for the total EAs in wheat was 0.229 mg/kg, and the EA values ranged from 0.012 to 0.666 mg/kg. The mean EAs values in Durum and barley were 0.225 and 0.065 mg/kg, respectively (Tittlemier et al., 2015).

A German study tested for the presence of EAs in different rye samples by developing a method using Liquid Chromatography with tandem mass spectrometry (LC-MS/MS). Bürk et al. (2006) found that ergometrine and ergocristine showed the lowest recovery percentage compared to the other individual alkaloids in rye bread and pumpernickel samples. For rye bread rolls,14 out of the 23 samples contained EAs above 10 μg/kg, and the highest alkaloids content observed in mixed-grain bread was found in Roggenmischobrot, which had 258 μg/kg of EAs. With the pumpernickel samples, the maximum EAs level presented in 1out of the 20 samples was 47 μg/kg, which was less than the warning level of 2,294 μg/kg reported for this product. In rye-crispbread, only 1 out of 14 samples contained a high level of total EAs (28 μg/kg). Finally, in rye bread-roll experiment, three of nine samples contained 11, 31, and 91 μg/kg alkaloids content. The study emphasized the need for market control regrading EAs, especially with rye breads (Bürk et al., 2006).

Topi et al. (2017) studied the EAs concentration for wheat samples collected in 2014 (n= 35) and 2015 (n= 36) in Albania. For the 2014 samples, 48.6% of them were tainted with EAs, compared to 19.4% of the wheat samples collected in 2015. For the 2014 positive samples, the EAs level reached roughly 17.3–975.4 μg kg $^{-}$1, with a mean value of 337.2 μg kg $^{-1}$. Also, the mean EAs value for the 2015 positive samples was 106.3 μg kg $^{-1}$, and the concentration ranged between 10.3 and 390.5 μg kg $^{-1}$.

Debegnach et al. (2019) examined rye and wheat samples for the presence of EAs. The test included six major EAs in the R-form (ergometrine, ergosine, ergocornine, α-ergocriptine, ergotamine, and ergocristine) and their respective epimers in the S-form (ergometrinine, ergosinine, ergocorninine, α-ergocryptinine, ergotaminine and ergocristinine). There were 71 flour and bread samples, and Ultra-High-Performance Liquid Chromatography (UHPLC) was

used to analyze the samples. Of the 71 samples, 20 were flour, and 51 were bread. Of the 20 flour samples, 16 were milled wheat, and 4 were milled rye. Of the 51 bread samples, 39 were wheat, and 12 were rye. The result indicated that 87% of all the samples were tainted with one or more of the EAs or their respective epimers. In the rye samples (bread and flour), ergocristine and ergocristinine were frequently presented while, in the wheat samples (bread and flour), ergometrine and ergometrinine were frequently observed. However, the presence of the R-forms was higher than the S-forms (77 and 23%, respectively). The sum of all EAs revealed that rye products (flour and bread) ranged from 2.5 to 188.6 μg/kg while wheat flour ranged from 2.5 to 28.6 μg/kg. Wheat bread ranged from 2.5-1,142.6 μg/kg, with 4 whole-wheat bread samples containing more than 150 μg/kg, which according to the study, was higher than the maximum limit established for grain products that are meant for human consumption (Debegnach et al., 2019).

Ruhland and Tischler (2008) determined the EAs levels in livestock grain using acid-base for HPLC (High Performance Liquid Chromatography) analysis. The study detected the individual presence of ergometrine, ergotamine, α-ergocryptine, ergocornine, ergocristine, and their sum in livestock mixed feed or feed grain. The content of EAs was separated using HPLC with excitation and emission wavelengths at 254 nm and 418 nm, respectively. The experiment demonstrated recovery rates between 82-120% in the five EAs. Subsequently, the study analyzed 124 samples of livestock mixed feed or feed grain and found that 91% of the samples contained EAs in a range of 10 μg/kg to 4883 μg/kg. The median number of the positive samples for total EAs in mixed feed was 70 μg/kg compared to 54 μg/kg in grain feed. Within the grain feed samples, rye contained the highest median number (96 μg/kg) of total EAs followed by wheat (29 μg/kg), and triticale (25 μg/kg) (Ruhland &Tischler, 2008).

Fungicides Used to Control Fungi in General

Fungicide contains chemicals that inhibit the growth of fungi or other organisms in order to control plants diseases. The Fungicide Resistance Action Committee (FRAC), is an international organization that provides guidelines about how to use fungicides and how to reduce fungicide resistance. Each fungicide includes a FRAC code presented on the label, and the code contains numbers and letters (Mueller et al., 2020a). This study focused on three chemical groups associated with three codes: FRAC code 3, FRAC code 7, and FRAC code 11.

FRAC Code 3

Triazoles are under FRAC code 3, and they target C14-demethylation during sterol biosynthesis (FRAC, 2005). Sterols are found in many eukaryotic cells and can be synthesized by fungi and other organisms. Cholesterol is considered to be main sterol in humans and animals, while sitosterol [(24R)-24-ethyl cholesterol] is found in some plants (Benveniste, 2004). Sterols are important for a fungus as they regulate the cell membrane's permeability. As a result, preventing fungal development can be achieved by using fungicide inhibitors that target sterol biosynthesis. One such option is sterol biosynthesis inhibitors (SBIs). In the late 1960s, many companies invented SBIs, such as triazoles, pyridines, and piperazines, that were used to control plant diseases. There are some steps involved with sterol biosynthesis; however, these fungicides inhibit the C14-demethylase catalysis (Ziogas & Malandrakis, 2015).

With fungi, Demethylation Inhibitors (DMIs) disrupt the sterol C14-demethylation step by blocking lanosterol C14-demethylation, which is mediated by an enzyme known as cytochrome P450 that contains a hemoprotein. A nitrogen from the DMIs is attached to the heme iron of cytochrome P450 and prevents the oxygen from linking, thus cutting off oxygen to the lanosterol C14-methyl (Ziogas & Malandrakis, 2015).

FRAC Code 7

According to the FRAC, the carboxamides under FRAC code 7 trigger Complex II in fungal respiration (succinate-dehydrogenase, SDH; FRAC, 2005). Most fungi have an electron-transport chain (ETC) that contain Complexes I-IV in the mitochondria, and one of the roles of Complex II is to help with respiration (Duvenage et al., 2019). In general, the mitochondria produce adenosine triphosphate (ATP) by using the NADH (Nicotinamide Adenine Dinucleotide) and $FADH_2$ (Flavin Adenine Dinucleotide) derived from the tricarboxylic acid (TCA) cycle and the conversion of oxygen to water (Rutter et al., 2010). Succinate dehydrogenase possesses a catalytic activity in the TCA cycle that specifically participates in the oxidation of succinate to fumarate (Cecchini, 2003). The produced electrons are transferred to the ubiquinone pool by the SDH enzyme (Sierotzki & Scalliet, 2013). Therefore, Complex II is also known as succinate: quinone oxidoreductase (SQR; Cecchini, 2003). The chemical classes of FRAC code 7 are known as a carboxamide fungicide (FRAC, 2005) and can disturb the TCA cycle in the mitochondria by inhibiting the SDH enzyme (Scalliet et al., 2012). SDHIs are used with plant disease due to their ability to attach to the ubiquinone binding (Q_p) site, which is located between the SDHB, SDHC, and SDHD subunit proteins of the SDH enzyme (Duvenage, et al., 2019; Sierotzki & Scalliet, 2013).This disruption, will prevent the reduction of ubiquinone at that site (Scalliet et al., 2012). In addition, this will obstruct any access to the SDH enzyme, thus disrupting the oxidation of succinate to fumarate (Sierotzki & Scalliet, 2013). The changes in the SDH enzyme cause greater amount of succinate and higher oxygen toxicity (Moreno et al., 2020). Also, inhibiting the Q_p site causes Complex II to form reactive oxygen species (ROS) as a result of the leaked electrons (Kluckova et al., 2015).

FRAC Code 11

The chemical group for FRAC code 11 targets Complex III of fungal respiration, specifically ubiquinol oxidase or the ubiquinol oxidation (Q_o) site. Quinone Outside Inhibitors (QoIs) fungicides are also known as strobilurin (FRAC, 2005). The Complex III structure contains three subunits, cytochrome c_1, cytochrome b, and the iron- sulfur protein, which are essential for the catalytic reaction to move electrons. Complex III encompasses two important sites: the quinol oxidation site, Q_o, and the quinone reduction site, Q_i. The cytochrome b subunit is in the center of the other subunits, and it can facilitate the movement of electrons from the Q_o site. In the inner membrane of the mitochondria, Complex III's catalytic reaction leads to the movement of electrons from ubiquinol; those electrons attach to the Q_o site and, eventually, to cytochrome c (Meunier et al., 2013). Bartlett et al. (2002) added that QoIs bind to the Q_o site, thus preventing the movement of electrons between cytochrome b and cytochrome c_1 as well as stopping the synthesis of ATP.

Fungicide Treatments for Ergot

In order to hinder ergot infection, growers spend approximately $14 to $35/acre/application on a number of fungicide treatments. There are a few fungicides available, and when repeating the same fungicide, the fungi might develop resistance. Therefore, new chemicals are needed to control ergot along with adequate knowledge about how and when to apply the fungicides (Kaur et al., 2018).

There are limited studies regarding the fungicide treatment's effect on ergot, and no study, until now, has reported the fungicides' effect on EAs content. The following studies described the effect of some fungicides on *C.purpurea* in field experiments that were conducted to control ergot disease. Puranik and Mather (1971) observed the effect of some fungicides'

effect on male-sterile barley inoculated with *C.purpurea.* The authors found that applying Benomyl (FRAC code 1) at a certain rate before and after anthesis decreased head infections by 29%, which means that the fungicide had not found its way to the ovary. Therefore, the researchers removed the glumes and applied the fungicides to ensure that the chemicals completely reached the ovary. Puranik and Mather (1971) reported that Benomyl reduced ergot infection in the floret from 83% to 5%, that Nabam decreased the infection to 30%, and that Dithane M-45 had a low control with floret infection.

Kaur et al. (2018) conducted a study to test the efficacy of four fungicide treatments on *C. purpurea* by using five fungicides—Priaxor, Solatenol, Fontelis, Propulse, and Quilt Xcel—on perennial ryegrass; the fungicide was applied during the time of anthesis. About 40 heads were collected from each of the replicated plots right after the production of honeydew, and the infected seed heads were counted. In addition, ergot severity and incidence were determined. Propulse, Priaxor, and Quilt Xcel had a significant effect for decreasing the incidence of honeydew produced during anthesis. Furthermore, all fungicides presented a significant effect on ergot severity, compared to the control, and these fungicides created similar effects as the "industry standard" fungicide, Quilt Xcel, with grass seed.

Cheng et al. (2017) observed nine fungicide treatments, applied at the flowering phase, on Kentucky bluegrass that was infected with *C. purpurea*. Samples were collected from 50 seed heads per plot. The researchers found it difficult to determine where there was a significant difference among the fungicides due to low incidence levels and the low severity of ergot presented in the plots. In spite of the low ergot incidence, ergot severity decreased by 50% when Priaxor was applied. In addition, Aproach, Luna privilege, Adepidyn, and Trivapro showed comparable effects to the standard fungicide, Quilt Xcel.

Dung et al. (2018a) observed the effect of the Priaxor SC, A19649B, Aproach 2.08 SC, Trivapro SE, and Quilt Xcel SE fungicides on two Kentucky bluegrass cultivars that were applied at the flowering stage. Then, 100 seed heads were collected from each cultivar, and the number of sclerotia from each panicle was determined to gauge the ergot's severity. The percentage of panicle was calculated to measure the incidence of sclerotia. In one type of cultivar, all the fungicides significantly decreased the ergot's severity and incidence, compared to the control, and none of the fungicides were different than the standard Quilt Xcel for grass. With the other cultivar, all fungicides significantly decreased the ergot's severity and incidence compared to the control.

Dabkevičius and Rsemaškienė (2002) also examined the effect of 10 different types of chemical seed dressers, the *Pseudomonas aureofaciens* bacterium, and the *Trichoderma lignorum* fungus on rye grain that was mixed with sclerotia from *C. purpurea*. After separating the sclerotia, the sclerotia were treated with the 10 chemical seed dressers, *P. aureofaciens* and *T. lignorum* treatments, and non-treated sclerotia. About 20 sclerotia (treated + control) were placed in five lines of rye fields at a certain soil depth. The ascocarp formation and sclerotia germination were observed at the commencement of anthesis and toward the end of anthesis. All chemicals, except the Tebeconazole *T. lignorum*, and *P.aureofaciens* treatments, significantly reduced the percentage of ergot germination when compared to the control. All these chemicals, except the *P.aureofaciens* and *T. lignorum*, significantly reduced the number of ascocarps that developed from the germinated sclerotia, compared to the control.

Dung et al. (2018b) aimed to control ergot germination and ascospore production by applying fungicides to the soil rather than protecting the flowers from an artificial *C.purpurea*. infection. Two *in-vitro* lab experiments were established to assess capitula production and the

number of ergot-body germinations. Only two fungicides—azoxystrobin + propiconazole (FRAC codes 11 and 3) and picoxystrobin + cyproconazole (FRAC codes 11 and 3)—significantly reduced capitula production and ergot germinations in the replicated lab experiments. The plots were artificially infested with *C.purpurea* in mid-October for three seasons (2014-2016); then, the plots were treated with five fungicides. All five fungicides reduced capitula production; however, fluopyram + prothioconazole reduced capitula production in the three years experiments more than azoxystrobin-alone, azoxystrobin + propiconazole, and pyraclostrobin-alone (Dung et al., 2018b).

MATERIALS AND METHODS

Materials

Hard Red Spring-Wheat Survey Samples (2019-2020)

All HRS-wheat survey samples in 2019 and 2020 obtained from fields located in the Unites States. The survey samples were collected as part of an annual HRS-wheat crop quality survey. The harvested samples were from four different states: Minnesota (MN), Montana (MT), North Dakota (ND), and South Dakota (SD). The samples for this study consisted of those containing ergot from the grader samples of the 2019 and 2020 HRS wheat regional crop quality survey. Additionally, 20 samples from each year with no ergot present were included. There were a higher number of samples with ergot sclerotia present in 2019, thus the higher number of samples. The total number of survey samples used in this study from 2019 was 153, and the number of samples used from each state was n= 36 (MN), n= 13 (MT), n= 79 (ND), n= 25 (SD). In the harvesting year of 2020, the total number of samples used was 54, and the number of samples used from each state was n= 5 (MN), n=7 (MT), n= 39 (ND), n= 3 (SD) (Figure 4).

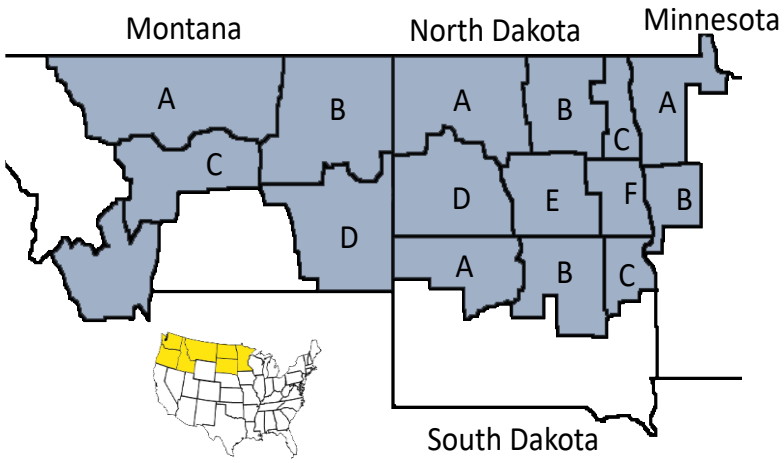

Figure 4. Regional map of HRS wheat samples obtained from the four growing states.

Ergot Sclerotia Sample Collections

A field experiment was established at the NDSU Agricultural Experiment Station-Fargo and designed as a randomized complete block with four replications in 2020. The samples were supplied by Dr. Andrew Friskop from the Department of Plant Pathology at NDSU.

Seven-row plots were sown with a confidential Hard Red Spring wheat cytoplasmic male sterile line (CMS-HRS wheat). The CMS-HRS wheat line was chosen as it is extremely susceptible to ergot (as it has an open stigma). Natural *C. purpurea* inoculum was used for the research site. Four fungicides and a non-treated control were evaluated in this experiment, and all fungicides were applied once at Feekes 10.5 (full-head stage). The four fungicides included: Miravis Ace (pydiflumetofen + propiconazole), Sphaerex (metconazole+ prothioconazole), Priaxor (fluxapyroxad + pyraclostrobin), and Quilt (propiconazole+ azoxystrobin) (Table1; Crop Protection Network, 2020). Individual plots were harvested with a plot combine and the entire sample was manually bagged. Each plot sample was then taken to a lab where the ergot sclerotia were separated from the other contents in the sample. Sclerotia were weighed, and then prepared for additional physiological and toxin analyses. Each sample was kept at 20 °C until further analysis.

Table 1. Four Fungicides applied to CMS-HRS wheat at Feekes 10.5 (full-head stage) and replicated four times.

Treatments	FRAC	Active ingredients	Mode of action	Rate A(fl oz)
Non-treated control	-	-	-	-
Priaxor	7+11	Fluxapyroxad + Pyraclostrobin	SDHI+QOI	8
Sphaerex	3	Metconazole Prothioconazole	DMI	7.3
Miravis Ace	3+7	Propiconazole+ Pydiflumetofen	DMI+SDHI	13.7
Quilt	3+11	Propiconazole Azoxystrobin	DMI+QOI	14.0

SDHI= Succinate- Dehydrogenase Inhibitor, QOI= Quinone Outside Inhibitor, DMI= Demethylation Inhibitor.

Methods

Hard Red Spring Wheat Survey Samples Quality Evaluations

Hard Red Spring Wheat Survey Samples Grading

The official United States standards for grain is determined by a licensed grain inspector. North Dakota Grain Inspection Service, Fargo, ND, provided grades for composite wheat samples representing each crop reporting area. Percent of shrunken and broken, foreign matter, damaged kernels, and total defects were determined during grading according to the USDA-FGIS standard procedures.

Percent of Dark, Hard, and Vitreous Kernels

A licensed grain inspector at the North Dakota Grain Inspection Service determine the percent DHV by approximating the percentage of kernels having vitreous endosperm.

Test Weight

Test weight was determined following the American Association of Cereal Chemists International (AACCI) Method 55-10.01. Samples were measured as pounds per bushel (lb/bu), kilograms per hectoliter (kg/hl) = (lbs/bu X 1.292) + 1.419. A clean sample of wheat passed inside a Boerner divider to diminish the size of the sample to 1 1/8 to 1 1/4-qt. Then, the sample was placed inside the hopper until a centered quart kettle is overfilled. Next, a stroker was held on the top of the kettle vertically and moved into three zigzag directions to eliminate excess kernels. Finally, the sample was transferred into a tared container, and the weight was recorded (Cereals and Grains Association, 2009).

Protein Content

In this study, protein content in wheat was measured utilizing Near-Infrared Reflectance (NIR) based on the AACCI Method 39-10.01. Samples were ground and placed in the machine

for testing. The wheat samples were expressed on dry basis and 12 percent moisture basis (Cereals and Grains Association, 2009).

Moisture Content

Moisture content was obtained following the Dickey-John Moisture Meter Official USDA procedure using Dickey-John Moisture Meter. The principle of this method is capacitance (dielectric constant).

Falling Number

The objective of falling number (FN) is to measure α-amylase activity during the breakdown of starch using an approved method of AACC International Method 56-81.03. Samples were ground, weighed to 7.00 ± 0.05 g on a 14% moisture basis, and placed into a tube. An aliquot of 25 ml of water at a temperature of 22± 2°C was pipetted into the tube. The tube was then closed with a stopper and shook for 20 to 30 times. Then, any remaining slurry around the interior of the tube was scraped down using a viscometer-stirrer. The tube along with the viscometer-stirrer were placed in a water bath, while the machine was started. The sample was stirred for 60 seconds before the viscometer-stirrers were dropped into the slurry. Finally, the time required for the viscometer-stirrers to fall was recorded. The calculation of the FN is based on the 14% moisture basis (Cereals and Grains Association, 2009).

$$FN(14\%\ \text{moisture basis}) = (FN_{as\ is}) \times (100-14)/(100-\text{moisture of sample, in\%})$$

Thousand Kernel Weight

Clean wheat samples (10 g), free of foreign material and broken kernels, were counted by electronic seed counter.

Wheat Dockage

Dockage was separated using official USDA procedure. An approved device (Carter Dockage Tester) was used to separate wheat samples from underdeveloped, shriveled, and small pieces of kernels.

Scanning Electron Microscope (SEM) Images of Ergot Sclerotia

Two random ergot sclerotia samples of each treatment were placed into a centrifuge tube and sent to the NDSU Electron Microscopy Center. Ergot sclerotia were scanned according to the method of Simsek et al. (2014) and Malalgoda et al. (2020). Ergot sclerotia were cut transversely across the center. A Hummer II sputter coater (Technics/Anatech Ltd., Alexandria, VA, USA) was used to coat the samples with a layer of gold. Images were taken by a JEOL JSM-6490LV Scanning Electron Microscope (SEM) (JEOL, Peabody, MA, USA) with 15kV voltage.

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Physical Characteristics of Ergot Sclerotia

Each fungicide treatment was replicated four times and physical characteristics measurements were taken for five sclerotia and the average values were recorded for each replicant. The physical characteristics included weight, width, and length. The weight was measured using Sartorius analytic scale, the width and length were measured using a Vernier caliper (Mitutoyo). Each treatment was placed in a plastic container. The weight of 10 ergot sclerotia from each treatment was taken 3 times by placing the ergot sclerotia in a weigh boat

and weighed in grams, and the three weighs were averaged. Afterward, five ergot sclerotia from each treatment were also measured for width and length with the Vernier caliper. After measuring the samples, they were placed in a watch glass, returned to their original bag, and stored for further analysis.

Compositional Characteristics of Ergot Sclerotia

Moisture Content of Ergot Sclerotia

The moisture of the ergot sclerotia samples was determined according to AACCI approved method 44-15.02. The sample (2-3 grams) was weighed and placed in an aluminum tin and set in a gravity oven at 130°C for one hour. After heating, the lids were placed back on the tins before being placed in a desiccator to cool. After cooling to room temperature, the tins were weighed, and the moisture was calculated based on the difference in sample weight before and after heating (Cereals and Grains Association, 2009).

% Moisture= (moisture loss in grams/original weight of sample)*100

Protein Content of Ergot Sclerotia

The purpose of protein analysis is to liberate nitrogen from the sample by a process of combustion utilizing intense heat. The procedure was done using LECO-FP-628 based on the AACCI Method 46-30.01. Pure oxygen was used for the combustion and Helium gas was used as a carrier gas. Ergot samples were weighed to 0.1 ± 0.05 g and encapsulated into a tin foil cup, twisted, and transported to the carousel autoloader. To free the Nitrogen, the samples were loaded inside a crucible, which is located inside the combustion tube. After the samples were burnt, the ash was collected inside the crucible. In the isolation system, the released Nitrogen was separated from other compounds and the detection system reported the results to percent

Nitrogen. Protein content was measured using the following equation (Cereals and Grains Association, 2009).

Crude protein %= %N x 6.25 is corrected for ergot

Ash Content of Ergot Sclerotia

Ash content was measured based on the AACC International Method 08-01.01. The ash dish was prepared by heating to eliminate any moisture, then placed into a desiccator to drop the temperature. After cooling, the ash dish was measured, and the value was noted. Each sample was ground and weighed to 3–5 ± 0.0001 g in the ash dish. Then, the dish containing the sample was placed inside a muffle furnace at room temperature. The sample was placed in the oven at 350 °C, after 1 h the oven was heated to 450 °C, and after another hour the temperature was increased to 590 °C. After a light ash was formed, the sample was transferred into a desiccator, allowed to cool, and weighed (Cereals and Grains Association, 2009). Ash percent was calculated as indicated below

% Ash= weight of residue/ sample weight*100

Analysis of *Fusarium* Toxins for HRS-Wheat Survey Samples and Ergot Sclerotia for CMS-HRS wheat Using Gas Chromatography-Mass Spectrometry (GC-MS)

Procedures

The analysis was done based on the method of Tacke and Casper (1996), with some modifications based on Mirocha et al. (1998). The samples were ground and a 2.5 g subsample was weighed into a 50 mL plastic centrifuge tube, and were extracted with 20 ml of acetonitrile: water 84:16 v/v. Samples were shaken on a Eberbach E6010 FIXED-SPEED RECIPROCAL shaker for 60 minutes, then the samples were placed on a cabinet and allowed to settle for 60 minutes. Four mL of the eluent from each sample was pipetted into a clean-up filter column

(1gm C18: alumina (1:3)) then captured into a glass cultural tube 16x100mm. After the gravity filtration step, 2 mL of the filtrate was pipetted into a screw cap tube and placed into Organomation Multivap Nitrogen Evaporator for 60 minutes at 55°C under compressed air steam to dryness. The samples were derivatized with 100 ul of TMSI: TMCS (1:1 v/v), vortexed for 10 seconds, and remained at room temperature for 30 minutes to react. A 1 mL aliquot of 0.5 ppm mirex internal standard solution was added, vortexed, then 1 mL of deionized water was added to each tube and vortexed for 10 seconds until the sample became translucent. Samples were placed on a horizontal shaker for 5 minutes, then allowed to settle for 15 minutes for separation of the two solutions. A glass Pasteur pipette was used for each sample to transfer the top layer inside the GC vials and were capped analyzed by Gas Chromatography-Mass Spectrometry (GC-MS) (GC 7890B with MS 5977B) Agilent (Agilent Technologies, Wilmington, USA). Samples were analyzed with three external standards and compared to a series of internal standards.

GAS Chromatogram

All Samples were injected at 1 ul into a spitless inlet at 300° C. The carrier gas used was helium; selected ion monitoring (SIM) determined the desired ions, and the values of the ion fragments were used to identify DON for HRS-wheat survey samples, and DON, NIV, FUX, 3ADON, DAS, NEO, HT-2,T-2, and ZER for ergot sclerotia.

Standard Curve

Standard curve was developed with DON free wheat matrix by adding 2 mL to 16 tubes. To each tube an aliquot of DON standard 0, 0.1, 0.2, 0.4, 0.5, 0.8, 1, 2, 3, 4, 6, 8, 10, 20, 30, 40 ng/ml was used to make standards of 0, 0.4, 0.8, 1.6, 2.4, 3.2, 4, 8, 12, 16, 24, 32, 40, 80, 120, 160 ppm DON, respectively. The same methodology was used to develop the standard curve for NIV, FUX, 3-ADON, 15-ADON, DAS, ENNS, HT-2, T-2, ZEN. The standard curve samples

were dried and derivatized in the same manner as the samples. Quantitative analysis for the regression curve was done by (MassHunter Workstation Software) to develop the standard curve equation ($y=ax^2+ bx+c$) that was used to calculate the concentration in the tube samples.

Ergot Alkaloids Analysis for HRS-Wheat Survey Samples and Ergot Sclerotia of CMS-HRS Wheat Samples

Prior to EAs determination, the HRS-wheat survey samples were ground using a Perten Lab Mill 3100 (Perkin Elmer, Waltham, MA, USA). Also, ergot sclerotia separated from the CMS-HRS wheat samples that were treated in the field, with Miravis Ace, Quilt, Priaxor, and Sphaerex and the non-treated control, were ground using a coffee grinder. After grinding, each sample was placed into a plastic bag, numbered, and stored at room temperature (21-24°C). The EAs content was measured using a Reveal Q+ Max for EAs test kit with a Raptor Integrated Analysis Platform (Neogen, Lansing, MI, USA). This test is a lateral flow quantitative method for determining total EAs (ergometrine, ergotamine, ergosine, ergocristine, ergocryptine and ergocornine with their respective enantiomers) in about 10 minutes. The principal of this method is based on antibodies pertained to EAs that are linked to colloidal gold particles. When EAs are present they will be trapped by the antibody-gold particle complex. Then, the particles concentrate and form a visible line after being wicked onto a space containing ergot alkaloid protein conjugates. Where less antibody-gold is caught in the test zone, the test line density decreases as the EAs concentration of the sample increases (Neogen, 2020).

The test was run according to the operating manual. The HRS-wheat sample was placed into an extraction cup and weighed to 10 g. Then, one pack of MAX 1 aqueous extraction packet was added to the extraction cup. Next, 50 mL of deionized water was dispensed into the mix of the extraction cup and shook for three minutes on an orbital shaker at 1,000 rpm. Then, the

sample was allowed to settle for at least 2 minutes. Afterward, 1mL of the extracted sample was pipetted inside a microcentrifuge tube and transferred into a centrifuge (Eppendorf 5415C) for 2 minutes at 14,000 rpm. Ergot sclerotia separated from the CMS-HRS wheat followed the same procedure for HRS wheat samples, but 0.1 g of the sample was weighed (Neogen, 2020).

Lateral Flow Raptor Integrated Analysis-Curve 1 (50-500 ppb)

Red sample dilution cup was placed inside a sample cup rack and labeled. After centrifugation, a 100 μL of the extracted sample was transferred into the red labeled dilution cup. Then, a 700 μL of the sample diluent was pipetted into the red dilution cup and dispersed by pipetting the mix 5 times slowly. Next, a test strip was placed inside the Raptor cartridge and transferred inside the Raptor port. After the bar code of the test strip was identified by the Raptor port, the sample ID was entered, and curve 1 was selected for 0-500 ppb. Finally, 400 μL of the extracted sample that was mixed with the sample diluent was transferred into the Raptor cartridge. After running the test, any sample appeared with a value of more than 500 ppb was followed another test for curve 2 (Neogen, 2020).

Lateral Flow Raptor Integrated Analysis-Curve 2 (500-5000)

In a new red dilution cup, a 200 μL of the mix (diluted sample) was drawn for a retest. Then, 800 μL of the diluent was pipetted into a dilution cup. The liquid was drawn and released slowly five times to mix. A new test strip was prepared in the same manner that was previously described but curve 2 was selected (Neogen, 2020).

Data Analysis

Analysis of Variance (ANOVA) was performed using the GLM procedure in Statistical Analysis System (SAS for Window 9.4, SAS Institute Inc., Cary, NC, USA.). The year and location combinations were considered as individual treatments and the HRS-wheat survey

samples as replicates of individual treatment in ANOVA. The ‘CONTRAST’ option was used to estimate significance of difference between mean values of growing seasons for locations. Least significant difference (LSD) values were generated using harmonized sample number at p= 0.05. Simple linear correlation coefficient was generated using the “CORR” procedure in SAS. Maximum, minimum, and median values were calculated using Microsoft excel version 16.30 (19101301).

For the fungicides’ experiment, the ANOVA was performed for all fungicides (combined) in relation to ergot sclerotia parameters using the SAS (SAS for Window 9.4, SAS Institute Inc., Cary, NC, USA.). The experimental layout was completely randomized block design with four replications. The LSD between fungicides were determined at P= 0.05 significance. Simple linear correlation was generated using the “CORR” procedure in SAS between ergot sclerotia parameters and EAs.

RESULTS AND DISCUSSION

Grading the Hard Red Spring Wheat Survey Samples

The HRS-wheat survey samples were classified into subclasses according to the USDA-FGIS' grain-grading primer: DNS, RS, and NS (USDA-FGIS, 2016). A licensed grain inspector determined the percentage of shrunken and broken kernels, foreign matter, damaged kernels, total defects, DHV kernels, and the test weight for the composite HRS-wheat survey samples representing each crop-reporting area. In general, most of the HRS-wheat samples collected from the four states in the two growing seasons were in the No.1 and No. 2 grades for HRS wheat, which indicates sound wheat, and possibly, a high yielding. During the 2019 growing season, HRS wheat samples were graded and classified as follows: n= 32 (1DNS), n= 87 (1NS), n= 15 (1RS), n= 1(2DNS), n= 9 (2NS), n= 2 (2RS), n= 3 (3DNS), n= 2 (3NS), n= 1(4DNS), and n= 1 (4RS). During the 2020 growing season, HRS wheat were graded and classified as follows: n= 19 (1DNS), n= 31 (1NS), n= 1 (1RS), n= 1(2DNS), n= 1 (2NS), and n= 1 (5DNS).

Variations of the Grading Factors for HRS-Wheat Survey Samples

Variation of the Test Weight

Test weight is one of the wheat-quality factors that assists with determining the kernels' soundness and the expected milling yield (Maghirang et al., 2006). Test weight is determined after the removal of dockage (USDA-FGIS, 2009). The test weight's maximum value was 63.66 lb/bu in the 2019 growing season and 64.25 lb/bu in 2020 (data not shown). The mean value for test weight was 60.2 lb/bu in the 2019 growing season, and 61.8 lb/bu in the 2020 growing season. This finding is close to a survey study conducted by Maghirang et al. (2006), who found an average test weight of 61.4 lb/bu in 98 samples of HRS wheat, and the test weight was 61.9 lb/bu when the sample (n= 75) was grouped with a protein content between 11.4% and 15.8%.

Table 2. F-values for the effect of growing condition (years and locations combined) on the quality parameters of HRS-wheat survey samples (2019 and 2020), including DON and EAs.

Traits[a]	Mean square	F-Value	Pr> F[b]
TWL	10.57	4.89	<.0001
DKG	0.61	0.67	0.8662
MC	4.09	7.11	<.0001
PC	3.38	3.74	<.0001
FN	5843.22	1.96	0.0078
TKW	32.95	3.60	<.0001
DHV	1225.54	2.91	<.0001
SAB	0.34	1.54	0.0636
FMT	0.11	1.11	0.3524
DMK	1.06	1.34	0.1487
TDF	1.86	1.59	0.0506
DON	10.43	2.47	0.0005
EAs	48521.35	2.48	0.0005

[a]TWL= Test weight per bushel; DKG= Dockage; MC= Moisture content; PC= Protein content; FN= Falling number; TKW= 1,000-kernel weight; DHV= Dark, hard, and vitreous kernels; SAB= Shrunken and broken; FMT= Foreign material; DMK= Damaged kernel; TDF= Total defects; DON= Deoxynivalenol; and EAs= Total alkaloids.
[b]Pr>F= probability (error df=183, model df=23).

Table 3. Mean and least significant difference (LSD) values for the wheat-quality parameters of HRS-wheat survey samples, including DON and EAs, naturally infected with *Fusarium* species and ergot *C. purpurea*.

Region	Year	N	TWL (lb/bu)	DKG %	MC %	PC %	FN Sec.	TKW g	DHV %	SAB %	FMT %	DMK %	TDF %	DON ppm	EAs [a] ppb
MN-A	19	25	60.2	0.8	13.2	14.7	382.2	37.2	59.2	0.4	0.0	0.4	0.8	1.2	33.2
MN-B	19	11	59.3	0.7	13.0	14.7	373.0	30.7	50.7	0.7	0.0	0.5	1.3	4.5	69.3
MT-A	19	3	58.0	0.8	11.8	16.2	390.7	33.5	98.0	0.9	0.0	0.0	0.9	0.0	0.0
MT-B	19	8	62.0	0.6	12.4	15.0	430.1	34.3	87.8	0.5	0.0	0.0	0.5	0.1	103.5
MT-C	19	1	57.7	0.3	11.6	14.6	361.0	36.0	48.0	0.9	0.0	0.6	1.5	0.1	121.9
MT-D	19	1	61.2	0.2	11.1	13.5	441.0	32.4	52.0	0.7	0.0	0.0	0.7	0.7	2.0
ND-A	19	17	61.2	0.4	13.0	15.1	394.8	33.9	61.4	0.5	0.0	0.1	0.6	0.4	73.1
ND-B	19	14	60.4	0.5	13.0	14.7	357.0	34.4	57.0	0.4	0.0	0.6	1.0	2.2	24.5
ND-C	19	11	60.6	0.9	13.1	14.1	340.8	34.2	56.9	0.4	0.0	0.3	0.7	0.6	78.9
ND-D	19	24	60.2	1.0	13.1	15.3	361.5	32.0	66.6	0.7	0.0	1.2	1.9	1.9	130.5
ND-E	19	8	61.0	1.1	13.2	15.4	360.3	32.5	71.3	0.5	0.0	0.6	1.0	1.0	163.1
ND-F	19	5	59.0	1.3	13.3	14.5	341.4	33.7	52.0	0.6	0.0	0.8	1.5	3.5	58.2
SD-A	19	7	60.5	1.0	12.9	14.5	384.4	32.8	44.0	1.2	0.0	0.1	1.3	1.1	71.7
SD-B	19	11	59.4	0.4	13.3	15.2	344.3	33.4	38.8	0.6	0.0	0.9	1.5	1.6	77.0
SD-C	19	7	59.2	1.0	13.2	14.6	323.4	34.0	44.9	0.5	0.0	0.2	0.7	1.9	199.6
MN-A	20	5	60.4	0.6	12.7	14.1	406.2	29.9	53.4	0.6	0.0	0.5	1.1	0.6	84.4
MT-A	20	1	63.8	2.0	11.5	11.6	381.0	29.2	78.0	0.5	0.0	0.0	0.5	0.0	0.0
MT-B	20	6	63.1	0.8	10.4	13.4	398.7	31.3	81.8	0.7	0.0	0.2	0.9	0.3	85.2
ND-A	20	15	62.5	0.7	12.6	14.7	399.9	34.2	65.5	0.4	0.1	0.7	1.2	0.6	212.7
ND-B	20	9	61.6	1.0	12.6	14.3	412.3	33.2	64.2	0.5	0.1	0.5	1.1	1.6	115.0
ND-D	20	14	61.2	0.9	11.3	16.1	393.9	31.4	64.9	0.8	0.4	0.6	1.8	0.2	278.0
ND-E	20	1	62.0	0.4	12.4	14.9	325.0	33.1	93.0	0.2	0.0	0.1	0.3	0.1	108.5
SD-B	20	1	60.9	0.7	12.8	15.7	406.0	31.2	60.0	0.2	0.2	0.7	1.1	0.0	374.4
SD-C	20	2	60.9	0.1	13.2	15.3	364.0	29.4	47.0	0.2	0.1	0.2	0.4	0.2	108.0
LSD[b] (P=0.05)	**-**	**-**	**2.3**	**NS**	**1.2**	**1.5**	**86.1**	**4.8**	**32.4**	**NS**	**NS**	**NS**	**NS**	**3.2**	**220.8**
CV%[c]	-	**-**	**2.4**	**122.9**	**5.9**	**6.4**	**14.5**	**9.1**	**33.6**	**83.9**	**716.8**	**171.2**	**96.3**	**159.7**	**130.4**

[a] N= Number of samples, TWL= Test weight per bushel, DKG= Dockage, MC= Moisture content, PC= Protein content(dry basis and 12% MB), FN= Falling number, TKW= Thousand kernel weight, DHV= Dark, hard, and vitreous kernels, SAB= Shrunken and broken kernels, FMT= Foreign material, DMK= Damaged kernels, TDF= Total defects, DON= Deoxynivalenol, and EAs= Total ergot alkaloids.

[b] LSD= Least significance difference at P= 0.05, NS= Not significant.

[c] CV= Coefficient of variance.

The analysis of variance for the combined data for locations and years indicated a significant ($P<0.05$) variation with the test weight (Table 2). Therefore, the growing conditions (year and location) had a significant influence on the test weight's variance.

In Table 3, the LSD indicated that the test weight for Montana (MT) region A in 2020 (only one sample obtained; 63.8 lb/bu) was significantly ($P\leq 0.05$) higher than the mean value for MT region A in 2019 (58.0 lb/bu). No significant ($P>0.05$) difference was found between the mean values of MT region B in 2019 (62.0 lb/bu) compared to MT region B in 2020 (63.1 lb/bu; Table3). Also, within the individual year of 2020, the test weight's value for MT region A (63.8 lb/bu) was significantly ($P\leq 0.05$) higher compared to Minnesota (MN) region A (60.4 lb/bu), North Dakota (ND) region D (61.2 lb/bu), and South Dakota (SD) regions B and C (60.9 lb/bu and 60.9 lb/bu, respectively). Across the growing years, MT region A in 2020 was significantly ($P\leq 0.05$) higher than all the regions during the 2019 growing season, except for MT region B (62.0 lb/bu). Also, within the individual year of 2019, MT region B (62 lb/bu) was significantly ($P\leq 0.05$) higher than other regions, such as MN region B, MT regions A and C, ND region F, and SD regions B and C in 2019.

McCaig et al. (2006) indicated that precipitation was a major contributor for reducing the test weight in wheat, and a gradual reduction of the test weight was observed between the 9^{th} and 12^{th} weeks of the ripeness phase due to rain. However, the mean values of the test weight for the samples obtained during the 2019 and 2020 growing seasons still met the minimum pound limit (58.0 lb/bu) for the U.S. grade No.1 HRS wheat, as established by the Federal Grain Inspection Service (FGIS; USDA-FGIS, 2014b), except for MT region C in 2019 (Table 3). The LSD indicated that no significant differences ($P>0.05$) appeared between the mean values for ND regions A, B, D, and E in 2019 compared to the corresponding North Dakota regions during the

2020 growing season (Table 3). However, within the individual year of 2019, significant differences were found between the mean values for some regions. For example, the mean value of ND region F (59.0 lb/bu) was significantly less than MT region B (62 lb/bu) in 2019. Also, ND regions A, B, and C in 2019 were significantly higher than MT region A in 2019 (Table 2). Across the growing years, some areas, such as SD regions C and B in 2019, were significantly lower than MT region A and ND region A in 2020 (Table 3).

The contrast analysis presented a probable year-and-location interaction effect (Table 4). To be specific, the results indicated a probable, significant ($P<0.05$) difference for the test weight between the 2019 and 2020 growing seasons for MT regions A and B (Table 4). Moreover, there was a probable, significant ($P\leq0.05$) year-and-location interaction effect with the test weight for ND regions A, B, D, and E in 2019 compared to the 2020 growing seasons (Table 4).

Overall, these results indicated that the locations showed inconsistent variations for the test weight between the growing years. As a result, there might be a significant interaction effect for the year and growing location on the test weight. In addition, locations had significant effects on the test weight within a year or across the growing seasons.

Table 4. Contrast analysis for the grading factors of HRS-wheat survey samples (degrees of freedom= 1).

Traits[a]	2019 vs 2020	Mean Square	F-Value	Pr> F[b]
TWL	MN A	0.01	0.05	0.8309
	MT A & B	29.39	13.59	0.0003
	ND A, B, D & E	12.90	5.97	0.0155
	SD B & C	6.42	2.97	0.0866
	MN, MT, ND & SD	42.96	19.87	<.0001
DHV	MN A	140.17	0.33	0.5645
	MT A & B	413.34	0.98	0.3229
	ND A, B, D & E	633.68	1.51	0.2213
	SD B & C	313.79	0.75	0.3889
	MN, MT, ND & SD	102.01	0.24	0.6230
SAB	MN A	0.26	1.16	0.2832
	MT A & B	0.02	0.11	0.7443
	ND A, B, D & E	0.04	0.19	0.6626
	SD B & C	0.24	1.06	0.3035
	MN, MT, ND & SD	0.14	0.62	0.4308
FMT	MN A	0.00	0.02	0.8982
	MT A & B	0.00	0.01	0.9347
	ND A, B, D & E	0.25	2.47	0.1179
	SD B & C	0.03	0.32	0.5748
	MN, MT, ND & SD	0.16	1.59	0.2088
DMK	MN A	0.04	0.04	0.8328
	MT A & B	0.03	0.04	0.8485
	ND A, B, D & E	0.12	0.15	0.6981
	SD B & C	0.04	0.05	0.8301
	MN, MT, ND & SD	0.03	0.03	0.8538
TDF	MN A	0.54	0.46	0.4978
	MT A & B	0.00	0.00	0.9687
	ND A, B, D & E	0.00	0.00	0.9632
	SD B & C	0.25	0.21	0.6464
	MN, MT, ND & SD	0.02	0.02	0.9020

[a]TWL= Test weight per bushel, DHV= Dark, hard, and vitreous kernels, SAB= Shrunken and broken, FMT= Foreign material, DMK= Damaged kernel, and TDF= Total defects.
[b]Pr>F= probability.

Variation of the Dark, Hard, and Vitreous Kernels (DHV) Kernels

Vitreous kernels are characterized as being translucent and spots-free (Baasandorj et al., 2015; McCaig et al., 2006). Vitreous kernels can be examined visually, but hardness can only be

tested mechanically (Samson et al., 2005). Baasandorj et al. (2015) found a significant correlation between protein content and DHV kernels for HRS wheat samples, and this association can be influenced by the growing condition. A high percentage of DHV kernels in HRS flour was also associated with a high water-absorption capacity, which is an essential factor for baking breads (Baasandorj et al., 2015).

In the present study, the total mean value for the DHV samples in 2019 was 59.3%, whereas it was 65.8% in 2020 (data not shown). During the 2019 growing year, the majority of 153 HRS wheat samples were classified as 1NS (n= 87) and 1DNS (n= 32). In the 2020 growing season, some of the 54 samples were classified as 1 DNS (n= 19) and other samples as 1NS (n= 31). The rest of the classifications for both years were previously listed in the grading section. This classification means that most HRS-wheat samples met the NS and DNS limits for the DHV kernels. The maximum and minimum DHV values for the 2019 samples were 99% and 5%, respectively, compared to maximum and minimum values for the 2020 samples: 96% and 20%, respectively, (data not shown). One explanation for the differences in the mean values between the growing years might be related to the nitrogen fertilizer. Samson et al. (2005) studied the fertilizer's effect on the Durum wheat's endosperm. They found that the development of kernel vitreousness is associated with nitrogen application during the flowering stage due to the increased of protein level.

The analysis of variance for the combined data for years and locations indicated a significant ($P<0.05$) DHV variation (Table 2), which means that growing conditions had a significant influence on the DHV variance. Also, the LSD showed a significant ($P\leq0.05$) difference for the mean values between locations within the same year or across both years (Table 3). For example, the mean DHV value for MT region A (98%) in 2019 was significantly

($P \leq 0.05$) higher than the mean values for MN regions A and B; MT regions C and D; ND regions A, B, C, and F; and SD regions A, B, and C during the 2019 growing season. Additionally, in 2019, the mean DHV value for MT region A was significantly higher than some areas, such as SD regions B and C; MN region A; and ND regions A, B, and D, in 2020 (Table 3). Similarly, in 2020, the DHV value for ND region E (only one sample obtained; 93%) was significantly ($P \leq 0.05$) higher than SD regions B and C, and MN region A, and across some regions, such as SD regions A, B, and C, in 2019. Another reason for the DHV differences in this study could be related to the varying weather among the states. McCaig et al. (2006) found reduced kernel vitreousness in response to severe rain during the 1989 growing season for Canadian Western Amber Durum wheat varieties.

Moreover, the contrast analysis in Table 4 showed no significant difference between years (2019 and 2020) for the individual states. This finding indicated that the influence of year or the year-and-location interaction might not be significant for the DHV with this HRS wheat sample set. In summary, locations had significant effects on DHV within the year or across the growing seasons for this experiment.

Variation of the Shrunken and Broken (SAB) Kernels

Shrunken and broken kernels are not only essential for the flour yield and grading, but are also important for the quality of the finished product (Afzal et al., 2012).The maximum percentage limit for SAB kernels in HRS wheat is graded with numeric values from 1 through 5, as determined by the U.S. standards for wheat (USDA-FGIS, 2014b). Afzal et al. (2012) found that shriveled kernels illustrated a significant elevation in the gluten content and the water-holding property compared to the healthy kernels while broken kernels illustrated a fewer gluten content and water-absorption property compared to the sound kernels. The total mean values for

the SAB kernels were 0.6% and 0.5 % during the 2019 and 2020 growing seasons, respectively (data not shown). These values suggested a low level of SAB kernels because they were less than the maximum level permitted for SAB kernels with grade No. 1 (3%) and No. 2 (5%), as determined by the U.S. wheat grades (USDA-FGIS, 2014b). These values indicated that there were very few SAB kernels presented in these regions. Sibbitt and Banasik (1973) conducted a study on HRS-wheat samples collected from different North Dakota counties and found an average value of 1.3 % in the final composite. In addition, the U.S. crop quality report for HRS wheat (version 2020) reported that the mean values for SAB kernels in the 2019 and 2020 growing seasons were similar (0.8%). The mean value for the SAB kernels in 2011 and 2012 was similar (1.1%; Simsek et al., 2013). The differences between the studies could be related to the temperature variances. Tashiro and Wardlaw (1990) indicated that a high temperature (36/31°C) at anthesis and 3 days post anthesis promoted shriveled kernels.

The analysis of variance for the combined data for location and year revealed no significant ($P>0.05$) variation for the SAB kernels (Table 2), meaning that the growing environment had no significant effect on the SAB kernels' variance. In addition, the LSD test found no significant ($P>0.05$) difference for the SAB kernels among locations. The result in Table 4 suggested a non-significant year-and-location interaction effect on the SAB kernels between the 2019 and 2020 growing seasons for the individual states. This result indicated that influences for the year or the year-and-location interaction might not be significant on the SAB kernels.

However, the highest mean value was found in SD region A (1.2%) for HRS wheat samples in 2019 (Table 2), and this amount was still less than the maximum-permitted limit for the top two SAB grades. It is be possible this elevated amount, although still meeting grade No.

1, might be related to another factor in that geographic area. Trematerra et al. (2000) discovered that, during grain storage, broken kernels that result from primary pests or from mechanically collecting the kernels in the field can attract secondary insects, causing more damage. Location and year had no significant interaction effect on SAB kernels. In addition, location had no significant effect on the variation of the SAB kernels.

Variation with the Foreign Material

Foreign material is the matter left after eliminating the wheat from dockage as well as shrunken and broken kernels, and the foreign material can be detected manually (hand sieve) or mechanically (USDA-FGIS, 2009). In 2019 and 2020, the total mean amount of foreign material was 0.0 % and 0.2 %, respectively (data not shown). The maximum mean values for the foreign material were 0.1% and 4.5% in 2019 and 2020, respectively. The maximum allowable limit for foreign materials, according to the U.S. wheat grade requirements for grades No. 1 and No. 2 is 0.4% and 0.7%, respectively (USDA-FGIS, 2009).

Fast et al. (2009) mentioned that in Oklahoma, foreign material between 1.1 and 5.0% is often discounted by grain dealers at an amount of 2.8 cents/hl. Mercier (1989) described the economic cost for wheat if dockage and foreign material were merged as one grading factor and then reported that the average values for the HRS wheat collected from different shiplots before export ranged from 0.23% to 0.40 % between the 1984 and 1988 growing years. Sibbitt and Banasik (1973) reported an average value of 0.3% for HRS wheat samples collected from different North Dakota regions. The results of the current study might indicate that, most foreign material was efficiently removed during dockage.

The analysis of variance illustrated that there was no significant ($P>0.05$) difference for the foreign matter, so the growing condition had no significant influence on the foreign matter's

variance (Table 2). The LSD had no significant (P>0.05) difference for foreign material among locations (Table 3). Also, the contrast analysis showed no significant difference between years (2019 and 2020) for the individual states (Table 4), indicating that the influence of year or the year-and-location interaction might not be significant for foreign material.

Overall, the results demonstrated that location and year had no significant interaction effect on foreign material. This result was probably because most mean values for the foreign matter were 0%. However, among the four states, ND region D had a 0.4% mean value for the foreign material during the 2020 growing season, which was still within the FGIS' established limit for grade No. 1 wheat (USDA-FGIS, 2009). In addition, location had no significant effect on the foreign material's variation.

Variation for the Damaged Kernels

Damaged wheat kernels are determined by characteristics such as: sprouted wheat, discolored kernels with purple pigment, damaged kernels due to scab or mold, tunneled kernels due to the presence of insects, undeveloped kernels that are green, and discolored kernels due to heat or fermentation (USDA-FGIS, 2009). Damaged kernels can lower the wheat's quality and can cause food-safety concerns (Kautzman et al., 2015).

The average values for the total damaged kernels in 2019 and 2020 were similar (0.5%; data not shown). This value is a good sign of sound wheat because the value is less than the maximum percentage level for the total damaged kernels that is allowed for U.S. grade No. 1 (2.0%) (USDA-FGIS, 2009). This result is consistent with the U.S. wheat-crop quality report for HRS wheat (version 2020), where damaged kernels for both years were below 2.0%; the average values for the damaged kernels of HRS wheat were reported as 0.0% and 1.0 % in 2020 and 2019, respectively (USW, 2020). However, the mean values for damaged kernels were slightly

higher than what was found in previous studies. Matthiensen et al. (1985) obtained a 0% mean value for the damaged kernels for HRS wheat in three different North Dakota district. Sibbitt and Banasik (1973) found an average damaged kernel of 0.2% in HRS wheat samples collected from different counties in North Dakota.

The analysis of variance for combined data revealed no significant (P>0.05) variation with the damaged kernels when the years and locations were combined (Table 2). The growing condition had no significant influence on the damaged kernels' variance. The LSD values had no significant (P>0.05) differences among locations for the damaged kernels (Table 3). Also, the contrast analysis showed no significant difference between year 2019 and year 2020 for the individual states (Table 4). This finding indicated that the influence of the year or the year-and-location interaction might not be significant for damaged kernels.

Beyer et al. (2007) found that environment (years) had a significant effect on the DON levels for the *Fusarium* damaged kernels. In Table 3, the highest mean value for the damaged kernels (1.2%) was found in ND region D in 2019, which corresponds to a mean value of 1.9 ppm of DON in that region. More details about the overall correlation are provided in the correlation section. Beyer et al. (2007) found that the winter wheat's genotype affected the DON concentration of the *Fusarium*-damaged kernels and that the *Fusarium*-damaged kernels for some cultivars were highly susceptible to DON than others. Therefore, it was possible that the HRS wheat genotypes played a role in ND region D. Overall, location and year had no significant interaction effect on damaged kernels. In addition, location had no significant effect on the variation of the damaged kernels.

Variation for the Total Defects

"Total defects" refers to the amount after adding up the shrunken and broken kernels, foreign material, and damaged kernels to obtain the total defects (Shelton & Martin, 2008), where the total defects cannot exceed the maximum percentage limit. The overall mean value of the total defects was 1.1% in 2019, and the mean value was 1.3% in 2020 (data not shown). There was a slight elevation for the mean value of the total defects during the 2020 growing season, but the measurement was still within the maximum limit (3.0%) for U.S. grade No. 1 wheat (USDA- FGIS, 2014b), which indicates sound and clean wheat. The maximum values for the total defects in the 2019 and 2020 growing seasons were 9.0 % and 5.8%, respectively (data not shown). Manthey et al. (2004) found an average total defect of 6.5 % in 178 samples of Durum wheat, which is higher than what was found in this study. Manthey et al. (2004) also indicated that, when the rate of FHB elevated, it caused an increased microbial load, thus raising the DON content. In the current study, the elevation of total defects in some areas could be related to FHB, ergot infection, or both. However, there was a difference in the weather between the two growing seasons. For example, during the 2020 growing season, rain was on time; the harvest was timely; and there was less FHB disease pressure in some areas (USW, 2020). On the other hand, in 2019, heavy rainfall hindered a timely harvest, and there was increased in FHB disease pressure (USW, 2019).

The analysis of variance for the combined data (locations and years) indicated no significant ($P>0.05$) variation in the total defects (Table 2), which implies that the growing conditions had no significant influence on the total defects' variance. Also, the LSD values had no significant differences ($P>0.05$) among locations for the total defects (Table 3). The contrast analysis showed no significant difference between years 2019 and 2020 for the individual states

(Table 4). The result indicated that the influence of the year or the year-and-location interaction might not be significant on the total defects for this HRS-wheat sample set.

Although there was no significant interaction effect for the total defects between the location and year, in general, it is important to be knowledgeable about the environment where the grain is growing in order to anticipate possible challenges related to biological hazards, diseases, pest control, and crop or soil stress (Awulachew, 2020). Overall, location and year had no significant interaction effect on the total defects. In addition, location had no significant effect on the total defects' variation.

Variations of the Non-Grading Factors for HRS-Wheat Survey Sample

Dockage Variation

Dockage is the process of removing non-wheat material based on weight, size, and shape; dockage does not affect the wheat's numerical grading (Shelton & Martin, 2008). The mean average for the dockage percentage removed in 2019 was 0.7%, and the value was elevated slightly to 0.8% in 2020 for the HRS-wheat samples (data not shown). This amount is lower than the result for a study conducted by Simsek et al. (2013), which found that the mean dockage percentages of HRS-wheat survey samples in 2011 and 2012 were at 1.1%.

The analysis of variance for the combined data from locations and years indicated no significant ($P>0.05$) variation in dockage (Table 2), meaning that the growing environment had no significant effect on the dockage's variance. In addition, the LSD test showed no significant ($P>0.05$) difference with the mean values among locations for dockage (Table 3). Also, the contrast analysis had no significant difference between years 2019 and 2020 for the individual states (Table 5). This finding indicated that influence of the year or the year-and-location interaction might not be significant for dockage.

However, the highest mean values for dockages were found in ND regions D (1.0%), E (1.1%), and F (1.3%); and SD regions A and C were at 1.0% for the 2019 survey samples. On the other hand, the 2020 survey samples also had high mean values for dockage. For example, the highest value for dockage was found in MT region A (only one sample obtained; 2%), and ND region B (1%; Table 3). Overall, location and year had no significant interaction effect on dockage. In addition, location had no significant effect on the dockage's variation.

Table 5. Contrast analysis for the non-grading factors of HRS-wheat survey samples (degrees of freedom= 1).

Traits[a]	2019 vs 2020	Mean Square	F-Value	Pr> F[b]
Dockage	MN A	0.14	0.15	0.6982
	MT A & B	1.23	1.37	0.2439
	ND A, B, D & E	0.00	0.00	0.9745
	SD B & C	0.29	0.32	0.5729
	MN, MT, ND & SD	0.05	0.05	0.8202
Moisture	MN A	1.44	2.51	0.1152
	MT A & B	3.34	5.81	0.0170
	ND A, B, D & E	7.34	12.77	0.0004
	SD B & C	0.12	0.21	0.6504
	MN, MT, ND & SD	8.83	15.36	0.0001
Protein	MN A	1.57	1.74	0.1886
	MT A & B	23.93	26.47	<.0001
	ND A, B, D & E	0.09	0.10	0.7578
	SD B & C	0.91	1.00	0.3177
	MN, MT, ND & SD	6.91	7.64	0.0063
FN	MN A	2392.01	0.80	0.3715
	MT A & B	1040.78	0.35	0.5553
	ND A, B, D & E	2137.19	0.72	0.3982
	SD B & C	6036.01	2.03	0.1564
	MN, MT, ND & SD	3953.38	1.33	0.2509
TKW	MN A	224.48	24.53	<.0001
	MT A & B	33.17	3.62	0.0585
	ND A, B, D & E	0.58	0.06	0.8010
	SD B & C	26.65	2.91	0.0896
	MN, MT, ND & SD	97.78	10.68	0.0013

[a]FN= Falling number and TKW= 1,000-kernel weight.
[b]Pr>F= probability.

Variation of the Moisture Content

Moisture content is a good indicator of the water's presence in wheat. The total mean values for the moisture content in 2019 was 13.0%, compared to 12.0% in 2020, for the HRS-

wheat samples (data not shown). These levels were higher than the mean values reported by Maghirang et al. (2006) for HRW wheat (10.6%) and HRS wheat (11.1%). Moisture content is a non-grading parameter for wheat, but it is a useful marker to determine the storage condition or during wheat processing (Maghirang et al., 2006). The overall mean values for the moisture content in this study, regardless of the year, were less than 14.5%, which implied good conditions for wheat storage. When storing wheat, a high moisture level (>14.5%) can lead to bacterial or fungal growth. As a result, high moisture can adversely affect the wheat's quality (Shelton & Martin, 2008). Most wheat is harvested at a safe moisture level to keep molds from growing. Therefore, if the wheat's moisture level exceeds 14.0-16.0%, the wheat or grains are dried to an acceptable percentage (El-Sisy, 2019).

The analysis of variance for the combined data (locations and years) found a significant ($P<0.05$) variation with the moisture content (Table 2). This result illustrated that growing conditions had a significant ($P<0.05$) influence on the moisture content's variance. The LSD indicated a non-significant ($P>0.05$) difference for the moisture content between MT region A in 2019 and MT region A in 2020 (Table 3). Also, the LSD showed a significant difference between the moisture content for MT region B (12.4%) in 2019 and MT region B (10.4%) in 2020 (Table 3). No significant differences were found for the mean values of ND regions A and B between the years. The only significant difference for the moisture content was found with ND region D between the years (Table 3). Also, there was a significant ($P\leq 0.05$) difference in the mean values among locations. For example, in 2019, the mean values for the moisture contents in ND region F and SD region B, both at 13.3%, were significantly ($P\leq0.05$) higher when compared to MT regions A, C, and D (11.8%, 11.6%, and 11.1 %, respectively) in the same year. In 2019, the moisture content for ND region F and SD region B was significantly ($P\leq0.05$)

higher than the moisture content for MT regions A and B, or ND region D during the 2020 growing year (Table 3). Within the year of 2020, the moisture content for SD region C was significantly ($P \leq 0.05$) higher than the moisture content for MT regions A and B, and ND region D in the same year. Also, in 2020, the moisture content for SD region C was significantly ($P \leq 0.05$) higher than the 2019 values for the moisture content in MT regions A, C, and D.

Storage length can affect the wheat's overall quality. For 6 months, Kibar (2015) observed changes in the moisture content of two wheat varieties inside silos. With one variety, the moisture content increased during storage until day 60 and then declined. The other variety had an increased moisture content up to day 90, with the value decreasing later. Changes in the moisture content during storage affected the test weight, and that situation may affect the flour yield (Kibar, 2015). During long storage, high temperature and relative humidity inside the silos affected the moisture content and other wheat parameters. These two factors are more important than the moisture content's initial value. The increase moisture content was associated with the air movement coming from the silo's window or door (Kibar, 2015).

Karunakaran et al. (2001) detected mold growth on wheat during storage when the moisture content ranged from 17%-19%. When the wheat's moisture contents was 15% and 16%, there was no mold present. Also, there was mold on wheat with a 19% moisture content when the samples were stored at 35 °C-20 °C; however, mold growth was not detected in the samples when wheat was stored at 15 °C and 10 °C during storage (Karunakaran et al., 2001). With wheat flour, insects become a problem with high moisture content (Nasir et al., 2003). Nasir et al. (2003) described how moisture levels of 9% and 10 %, measured after packaging, restricted the growth of molds or insects when using paper packaging; a 9% moisture content was more suitable when storing wheat flour for a longer time (Nasir et al., 2003).

The contrast analysis revealed a probable, significant year-and-state interaction effect for moisture content (Table 5). Specifically, there was a possible, significant difference when comparing MT regions A and B in 2019 to the same regions' 2020 HRS-wheat samples (Table 5). In addition, there was a probable, significant difference ($P \leq 0.05$) when comparing ND regions A, B, D, and E in 2019 compared to the same regions in 2020 (Table 5).

These results indicated that the locations had inconsistent variations for the moisture content between the growing years. As a result, there might be a significant interaction effect for the year and growing location on the moisture content. In addition, locations had a significant effect on the moisture content's variation across years or within the same year.

Variation with the Protein Content

Protein provides an insight about the wheat's end-use quality (Maghirang et al., 2006). Wheat, specifically HRS wheat, is known for its high protein content and its appropriateness to make breads. Therefore, protein content can be used to make a decision about the finished products (Simsek et al., 2013). The total mean value of the protein content for the HRS-wheat samples from the 2019 and 2020 growing seasons was similar (14.8%; data not shown). These mean values resembled Maghirang et al.'s (2006) findings. The authors found an average protein content of 14.6% in the HRS-wheat samples. In addition, the mean values for the protein content in both years of this study were similar to Simsek et al. (2013), who found mean protein values of 14.8% and 14.6% for 2011 and 2012 in HRS-wheat samples, respectively.

The analysis of variance for the combined data of locations and years revealed a significant ($P < 0.05$) variation for the protein content (Table 2), meaning that growing conditions had a significant effect on the protein content's variance. Furthermore, the LSD indicated that the protein content for MT regions A and B in 2019 was significantly ($P \leq 0.05$) higher than the same

regions in 2020 (Table 3). Also, there was a significant ($P \leq 0.05$) difference among locations within the individual year, or across the years. For example, the highest mean value for protein was found in ND region D (16.1%) in 2020, and that value was significantly higher than ND region B, MN region A, and MT regions A and B for the same year; in 2020, the protein content for ND region D was also significantly ($P \leq 0.05$) higher than the value for some regions, such as MT region D, ND regions C and F, and SD region A, in 2019. Also, the protein content for MT region A (16.2%) in the 2019 growing season was significantly ($P \leq 0.05$) higher than MN regions A and B; MT regions C and D; ND regions B, C, and F; and SD regions A and C. Across the 2020 year, the protein content for MT region A in 2019 was significantly ($P \leq 0.05$) higher than some regions, such as MN region A, MT regions A and B, and ND regions A and B, in 2020.

The contrast analysis given in Table 5 indicated that there was a probable year-and-location interaction effect for the protein content. Specifically, MT regions A and B had a probable, significant difference between the two seasons (Table 5).

One of the points discussed in Dupont and Altenbach's (2003) review about the effect of the environment on the wheat's protein content was the use of fertilizers and temperature, which can influence the protein's storage. Also, the aggregation of protein and starch is based on two factors; the number and the size of the endosperm cells found in the individual kernel. Additionally, temperature affects the days for wheat filling period (Dupont & Altenbach, 2003). Wieser and Seilmeier (1998) found that there was a high protein content for the wheat as the fertilizer levels increased. The higher protein levels were not only influenced by the increased level of nitrogen fertilization, but also by the wheat variety.

These results indicated that locations showed inconsistent variations for the protein content between the two growing years, meaning that there might be a significant interaction

effect for the year and the growing location on the protein content. In addition, the locations had a significant effect on the protein content's variation across years or within the same year.

Variation of the Falling Number

The falling number's purpose is to estimate, in seconds (sec), the alpha-amylase activity for a sample. A high level of alpha-amylase activity corresponds to a low falling number and sprouted wheat, which impact the wheat's end product, causing problems when trading grain inside or outside the United States (USDA-FGIS, 2019).

A low falling number, caused by increased alpha amylase activity, can affect growers. In general, a common reason for the decreased falling number is preharvest sprouting, but the late maturity of alpha amylase could be another reason. Also, a high falling number of more than 250 sec, which is determined by the receiving country, is necessary for a high-grade wheat (Mares & Mrva, 2008). A falling number between 250 sec and 275 sec is an indication of sprouted wheat, resulting in a decreased price and grade for the wheat; however, debranning the wheat prior to milling raised the falling number (Hareland, 2003). In addition, increased alpha-amylase activity can cause issues during storage and can impact the final products' quality (Mares & Mrva, 2008). Kiszonas et al. (2018) explained that there was a moderately inverse correlation between the falling number and the alpha-amylase activity for flour, but the association was not strong.

In the current study, the total mean value for the falling number in 2019 was 370 sec compared to 398 sec in 2020 (data not shown). This finding indicated that the HRS-wheat samples from both years were sounds with a slight increase for the falling number with the 2020 HRS-wheat samples. The analysis of variance for the combined data (locations and years) presented a significant ($P<0.05$) variation for the falling number, implying that the growing environment had a significant effect on the falling number's variance (Table 2).The LSD

illustrated that there was a significant ($P \leq 0.05$) difference with the mean values for the falling number among some locations within the individual year, or across the growing years. The highest mean value for the falling number during the 2019 growing season was found in MT region D, with an average falling number of 441.0 sec, which was significantly ($P \leq 0.05$) higher when compared to ND regions C and F, and SD regions B and C in the same year. The falling number for MT region D in 2019 was higher than ND region E in 2020 (Table 3). For the 2020 growing season, the highest mean value for the falling number was found in ND region B (412.3 sec), which was significantly ($P \leq 0.05$) higher than the falling number for ND region E in 2020 or SD region C in 2019 (Table 3). The contrast analysis for falling number in Table 5 showed no significant difference between the years (2019 and 2020) for the individual states (Table 5). This finding indicated that the influence of the year or the year-and-location interaction might not be significant for the falling number. The results for the falling number in this experiment showed that only the location had a significant effect on the falling number's variation across years or within the same year.

Variation of the Thousand Kernel Weight

Thousand kernel weight (TKW) is used to determine the seed's mass. TKW is also associated with the wheat's yield (Xu et al., 2019). The total mean value for the TKW with the 2019 samples was 33.8 g, compared to 32.2 g in 2020 (data not shown). The total mean value for the TKW in 2019 was slightly higher than the total mean value from the 2020 growing season. In addition, the total mean value for the DON content was higher in 2019 (1.5 ppm) than in 2020 (0.6 ppm). Xu et al. (2019) conducted a study with 370 wheat samples that were collected from different regions in China, and the researchers found different TKW levels in many regions, with a mean value of 42.6 g for the 2015 wheat samples, which was higher than our study. Xu et al.

(2019) also observed a decrease with the TKW due to a high DON content. In 2015, China had a severe *Fusarium* infection in its Anhui province, and the mean value for the DON content was high (17,753.8μg/kg) compared to our study:1.5 ppm in 2019 and 0.6 ppm in 2020. The different DON contents among the regions could be related to the variations with the weather, wheat resistance to DON, and FHB species (Xu et al., 2019).

The analysis of variance for the combined data (locations and years) indicated that there was a significant ($P<0.05$) variation for the TKW (Table 2), meaning that the growing environment had a significant effect on the TKW's variance. The LSD illustrated that the mean TKW value for MN region A in 2019 was significantly ($P\leq 0.05$) higher, with a 7.3 g difference, compared to MN region A in 2020 (Table 3). In addition, the LSD suggested a significant ($P\leq0.05$) difference with the mean TKW values among locations within the individual year or across the years. For example, the TKW for MN region A in 2019 was significantly ($P\leq0.05$) higher than the TKW for MN region B, MT region D, and ND region D in 2019. Also, the TKW for MN region A in 2019 was significantly higher than the TKW for MT regions A and B, ND region D, and SD regions B and C in 2020. Within the individual year of 2020, the mean value for the TKW in ND region A (34.2 g) was significantly ($P\leq0.05$) higher than the TKW values for MT region A and SD region C in the same year; no significant difference was found across the 2019 year.

The contrast analysis suggested a probable, significant year-and-location interaction effect for the TKW (Table 5). Specifically, there was a probable, significant ($P<0.05$) difference between the years of 2019 and 2020 for MN region A (Table 5).

Many reasons for the different 1,000-kernel weights were discussed in the previous literature. Baasandorj et al. (2015) found that the mean TKW for the 2010 growing season was 4

g higher than with the 2011 samples and explained that the TKW reduction was related to fewer planted HRS-wheat acres and high rain during the spring. Mohammadi (2012) explained that heat could reduce the TKW by 23.7 % with warm conditions. During heat stress, Mohammadi observed a reduced in the TKW (about 14.9%) from the time of anthesis to the time of ripeness "per degree centigrade increase in mean temperature" (Mohammadi, 2012, p. 2933). Disease pressure can also decrease the TKW. Siuda et al. (2010) found a mean TKW reduction of approximately 30% with kernels that are severely infected with *F. culmorum*, compared to about a 13% reduction for medium-and low-infected kernels.

In summary, the results indicate that the locations showed inconsistent variations with the TKW between the two growing years, so there might be a significant interaction effect for the year and the growing location on the TKW. In addition, the locations had a significant effect on the TKW variation within an individual year.

Variation of the Deoxynivalenol Content for HRS-Wheat Survey Samples

In 2019, the total mean value for the DON content was 1.5 ppm compared, to 0.6 ppm in 2020. These values, in general, were less than the maximum DON level (2.0 ppm) in unfinished cereal grains that were intended for further processing (CAC, 2019). However, the total mean value for DON content in 2019 was higher than the total mean value from the 2020 growing season. This finding is possibly related to rain spreading across these regions, which resulted in increased FHB pressure (UWS, 2019), compared to a less disease pressure in 2020 (USW, 2020).

These mean values were less than what was reported in a study by Xu et al. (2019), who found an average of 17,753.8 μg/kg in 370 wheat samples infected with *Fusarium* that were collected from China's Anhui province during the 2015 growing season. The mean DON levels reported by Tittlemier et al. (2013) were between 0.06 and 1.3 mg/kg for Canadian Western

Amber Durum wheat collected in 2010 from different regions of Saskatchewan. A mean value of 478 μg kg^{-1} was found in 29 wheat samples collected from two regions in Hungary (Tima et al., 2016), which was less than this study.

The analysis of variance for the combined data (locations and years) illustrated a significant ($P<0.05$) variation for the DON content (Table 2). This finding suggested that the growing condition had a significant influence on the DON variance. The LSD showed a significant ($P\leq0.05$) difference for the mean values between locations. For example, within the individual year of 2019, the mean value for MN region B (4.5 ppm) was significantly ($P\leq0.05$) higher than the mean value for MN region A; MT regions A, B, C, and D; ND regions A, C, and E; and SD region A in 2019. Also, in 2019, the mean value for MN region B continued to be significantly higher when compared to all regions, except for ND region B, in 2020 (Table 3). Also in 2019, the mean value for ND region F was significantly ($P\leq0.05$) higher than the mean value for MT regions A, B, and C; the mean value for ND region F in 2019 was also significantly ($P\leq 0.05$) higher than the mean value for MT regions A and B, ND regions D and E, and SD regions B and C. No significant difference was found between the mean values for the DON content among locations within the individual year during the 2020 growing season (Table3). The contrast analysis showed no significant difference between the years of 2019 and 2020 for individual states (Table 6). This illustrated that the influence of the year or the year-and-location interaction might not be significant for DON content.

Table 6. Contrast analysis for DON and the total EAs of the HRS-wheat survey samples (degrees of freedom= 1).

Toxins[a]	2019 vs 2020	Mean Square	F-Value	Pr> F[b]
DON	MN A	1.94	0.46	0.4990
	MT A & B	0.02	0.00	0.9439
	ND A, B, D & E	5.76	1.36	0.2444
	SD B & C	6.35	1.50	0.2216
	MN, MT, ND & SD	8.98	2.13	0.1464
EAs	MN A	10962.81	0.56	0.4554
	MT A & B	204.99	0.01	0.9186
	ND A, B, D & E	67442.92	3.44	0.0652
	SD B & C	24427.77	1.25	0.2657
	MN, MT, ND & SD	61336.44	3.13	0.0785

[a]DON= Deoxynivalenol and EAs = Total ergot alkaloids.
[b]Pr>F= probability.

To sum, during the 2019 HRS wheat growing season, MN region B (4.5 ppm), ND region B (2.2 ppm), and ND region F (3.5 ppm) exceeded the maximum DON level (2.0 ppm) for unfinished cereal grains that are intended for further processing (CAC, 2019). This high amount is possibly due to the 2019 rain that resulted in an increased FHB pressure (USW, 2019). According to the Minnesota Department of Natural Resources (2022), Minnesota received more heavy rain in 2019. According to the *North Dakota Annual Climate Summary*, the precipitation in North Dakota during 2019 was 11.47 inches higher than 2020 (North Dakota State Climate Office, 2020).

Xu et al. (2019) mentioned that possible variations for the DON levels in different regions of the Anhui province might be related to the variable toxicity of the fungal species, plant resistance to *Fusarium*, or weather. In addition, FHB infection or DON production in the grains is affected by wet weather, temperature, and relative humidity (Wegulo, 2012). Cowger et al. (2009) found a significant increase with the DON content for Soft Red Winter wheat exposed to different wet days, ranged from 0, 10, and 20 days, after anthesis. Cowger et al. concluded that

the mist days' duration after anthesis should be considered as a marker when assessing DON as well as temperature and rain. Stanciu et al. (2019) studied 105 wheat samples in multiple regions of Romania with different climatic conditions and found that the DON content tends to increase when there is rain toward the last stage of flowering, dry days during grain development, or more moisture right before harvest. Stanciu et al. (2019) also implied that precipitation had a more profound effect on DON when compared to temperature.

However, Popovski et al. (2017) conducted a study in Slovenia; they examined naturally infected wheat varieties with FHB in two different locations and two growing seasons (2012 and 2013). One location tended to be humid, had higher infection levels of *F. culmorum* and *F. graminearum*, and had a higher DON content compared to the other location. The authors found no correlation between the incidence of FHB and weather (temperature and rainfall) during the wheat's flowering stage, thus weather had no impact on the mean levels of *Fusarium* infection. They suggested that, in addition to the environment, other variables could cause the infection (Popovski et al., 2017).

Alkadri et al. (2014) studied the level of mycotoxins in wheat that was collected from Italy and Syria, the authors reported that most of the samples were tainted with different types of mycotoxins, Alkadri et al. (2014) also suggested that weather, agronomic factors, and the growing years all played a role in modifying the fungal mycoflora. In a review, Wegulo (2012) discussed many factors that affect the grain's FHB and DON levels. Nitrogen, wind, and rain can contribute to lodging, which increase the DON content. Another mentioned factor was the practice of conservation tillage, which is leaving crop debris in the field. This action can improve the soil's quality and can help maintain good yield; however, this approach can be lead to pathogen growth (Wegulo, 2012). Crop rotation, such as growing soybeans prior to wheat, may

reduce the DON content when compared to growing maize prior to wheat (Beyer et al., 2006; Wegulo, 2012). Although using a fungicide decreased the FHB and DON content for many research grains, particularly triazols (Beyer et al., 2006; Wegulo, 2012), the use of strobilurins can increase the DON content level (Wegulo, 2012). Location had a significant effect on the DON variation for HRS-wheat survey samples within the 2019 growing season.

Variation of the Ergot-Alkaloids Content for HRS-Wheat Survey Samples

Ergot contamination can create concerns throughout the supply chain. For example, infected grain can cause a problem for growers because this situation can affect the grain's yields and grades. Feed manufacturers reject the production of contaminated feed because it can affect the animals' health and performance. Also, contaminated food can be toxic to humans (Young, 1981). Mitchell and Cooke (1968) found that sclerotia which were stored at 15 °C for 12 months could germinate. The total mean value for the total EAs in HRS-wheat samples in 2020 was 180.5 ppb, compared to a mean value of 81.5 ppb during the 2019 growing season (data not shown). The maximum EAs levels were 485.0 ppb and 657.5 ppb (data not shown) in 2019 and 2020, respectively.

The mean value for the total EAs in 2020 was higher than the current limit that is recommended by the European Union (EU), which is 150 ppb for milling products with an ash content above 0.9% for whole-grain flour (Official Journal for the European Union, 2021). The current study presented higher EAs levels for the HRS-wheat survey samples than for the whole-wheat flour or the rye flour observed in a study by Debegnach et al. (2019), who found that the sum of all EAs for the rye products ranged from 2.5 μg/kg to 61.3 μg/kg for rye flour and from 2.9 μg/kg to 188.6 μg/kg for rye bread. The sum of the EAs for whole-wheat flour was from 2.5 μg/kg to 28.6 μg/kg, and the value for wheat bread ranged from 2.5 μg/kg-1,142.6 μg/kg

(Debegnach et al., 2019). Also, the mean values for both years in the present study were less than what was observed by Babič et al.'s (2020) examination of wheat and rye. Babič et al. (2020) collected grain samples in Slovenia from 2014 to 2017 and analyzed the total EAs using LC-MS/MS. Of the 206 wheat samples, 34 of them were positives for EAs; the mean value for the total EAs was 363 μg/kg, and the maximum value was 4,217 μg/kg. These wheat values were higher than the mean and maximum levels found in our study's HRS-wheat survey samples for both years. With the rye samples (n= 136), 19 of them were positives, and the mean and maximum level values were 502 μg/kg and 4,114 μg/kg, respectively.

Ruhland and Tischler (2008) analyzed 124 samples of livestock mixed feed or feed grain, finding that 91% of the samples contained EAs (total of ergometrine, ergotamine, ergocornine, α-ergocryptine and ergocristine) in a range of 10 μg/kg to 4,883 μg/kg. For the total feed-grain samples (n= 64), the median number of the samples was 54 μg/kg, which was higher than the median value in 2019 (15.90 ppb) and was less than the median value for 2020 (114.70 ppb) in this study (data not shown). Ruhland and Tischler (2008) also found that, within the feed-grain samples, rye (n= 15) contained the highest median value (96 μg/kg) of EAs, and the maximum value was 1,067 μg/kg. For wheat (n= 21), the median and maximum EAs values were 29 μg/kg and 1,236 μg/kg, respectively. For triticale (n=14), the median and the maximum EAs were values were 25 μg/kg and 1,103 μg/kg, respectively. These maximum values were higher than the maximum amount of EAs for both years in the current study (485.0 ppb and 657.5 ppb in 2019 and 2020, respectively).

Tittlemier et al. (2015) found higher total EAs values for harvest samples of wheat and Durum (<limit of quantification (LOQ) to 7.81 mg/kg) than the shipment samples. Among the shipment samples, the mean value of the total 10 EAs for the wheat samples was 0.229 mg/kg,

ranging from 0.012 mg/kg to 0.666 mg/kg; these mean values were greater than the mean value reported in the current study for both years.

Also, the mean values for the total EAs (sum of ergometrine, ergometrinine, ergosine, ergosinine, ergocornine, ergocorninine, ergocryptine, ergocryptinine, ergotamine, ergotaminine, ergocristine, and ergocristinine) in the positive wheat samples, taken from Albania and reported by Topi et al. (2017), were 337.2 μg kg^{-1} and 106.3 μg kg^{-1} in 2014 and 2015, respectively. Shi et al. (2019) found higher mean values (1,150.5 μg/kg) for the total EAs (ergocornine, ergocristine, ergocryptine, ergometrine, ergosine, and ergotamine) in 49 of 67 barley samples in Canada when compared to the means values for the total EAs in both years of this study.

One reason for the varying averages between the two growing seasons in the current study, or when compared to other studies, could be explained by the storage period. The 2020 survey samples were newer compared to the 2019 samples. The 2019 HRS-wheat survey samples were stored for more than 1 year before starting the analysis in 2021.Tittlemier et al. (2015) described a similar situation with storing the shipment samples for over a year before analysis. The 2011 harvested samples were newer than the shipment samples. The mean values for the total EAs fractions were higher with the *R* epimers, but the shipment samples contained higher *S* epimers; as a result epimerization could change the *R*-forms into *S*-forms (less-toxic compounds). Tittlemier et al. (2015) also mentioned that the higher EAs levels in the harvested samples might be related to the grains being uncleaned (The ergot bodies were not removed.) compared to the shipment samples. However, the current study did not measure the individual EAs. This situation was also discussed by Blaney et al. (2009), who found that the total EAs (ergotamine, ergocornine, a-ergocryptine, ergocristine and ergonovine) for rye-ergot sclerotia

samples stored for years had no remarkable difference compared to the samples stored for 3 months. The authors also found that the percentage of the *S* enantiomers in the old samples was higher (36%) than it was with the newly stored samples (20%); however, the current study did not investigate the individual EAs, only the total EAs. Ergopeptinines, the respective epimer of ergopeptine, can be form during long and poor storage for grain (Krska & Crews, 2008).

Other factors, such as the use of fungicides with strobilurins and azoles, can hinder conidial and mycelium developments, respectively. "Agronomic practice", such as crop rotation by planting hosts that are less vulnerable to ergot infection, can decrease this disease in the field because ergot bodies cannot thrive for more than 12 months (Miedaner & Geiger, 2015). Miedaner and Geiger (2015) explained that one factor which affects ergot infection is host resistance. Wheat, barley, and sorghum are self-pollinators and have a lower chance of ergot infection, compared to rye, because their florets remain unopened.

Also, the total EAs in the current study had high mean values when compared to some studies but were less than the amount reported in other previous studies, and this result was due to many factors. The current study utilized lateral flow test (Reveal Q+ Max kit for EAs) to detect EAs in HRS-wheat survey samples, and previous studies used various methods and different grain samples to measure the total EAs. For example, Tittlemier et al. (2015) used the UPLC-MS/MS method; while Topi et al. (2017) and Babič et al. (2020) utilized the LC-MS/MS method; and Ruhland and Tischler (2008) used the HPLC method. Also, the EAs extraction procedures utilized with the previous studies were different than the current study. The type of total EAs detected was similar to Topi et al. (2017) and Babič et al. (2020), but was different than what was reported by Tittlemier et al. (2015) and Ruhland and Tischler (2008).

The analysis of variance for the combined data (locations and years) showed a significant ($P<0.05$) variation for the EAs (Table 2). This finding suggested that the growing conditions had a significant influence on the total EAs' variance. Specifically, the 2020 sample from SD region B contained the highest amount of total EAs, with a value of 374.4 ppb (only one sample obtained; Table 3). Therefore, in 2020, the amount of total EAs for SD region B was significantly ($P\leq0.05$) higher than SD region C, ND regions B and E, MT regions A and B, and MN region A. In addition, the amount of total EAs for SD region B (374.4 ppb) in 2020 was significantly higher than the mean values for MN regions A and B; MT regions A, B, C, and D; ND regions A, B, C, D, and F; and SD regions A and B for the 2019 growing season (Table 2). Also, the mean value for total EAs in ND region D (278.0 ppb) was significantly ($P\leq0.05$) higher than the amount of EAs for MT region A in 2020 and was also significantly higher than MN region A, MT regions A and D, and ND region B during the 2019 growing season (Table 3).

No significant difference was found between locations within the individual year for the 2019 growing season (Table 3). Also, the contrast analysis showed no significant difference between the years of 2019 and 2020 for the individual states (Table 6). This result suggested that the influence of the year or the year-and-location interaction might not be significant for the total EAs content.

It is difficult to explain why some regions had more than 150 ppb of total EAs for both years, but this increase could be related to location, agricultural practices, or weather conditions. Young (1981) found that the percentage for the total EAs content in Canadian rye ergot that was collected from the Prince Edward Island region (n= 10) was significantly less compared to the other three regions. Babič et al. (2020) found a significant difference with the mean value for the total EAs between the years. In the 2014 samples, the mean value for the total EAs was

significantly higher than it was in 2015, 2016, or 2017. Babič et al. (2020) noted that Slovenia's precipitation in 2014 was significantly higher compared to the average from 1981-2010. Mainka et al. (2007) conducted a study with different varieties of rye-ergot samples that were artificially inoculated with *C. purpurea* at three different locations in Germany during 2002, 2003, and 2004. The authors found that the total EAs were significantly influenced by the year ($P<0.001$). The researchers also examined the location factor and indicated that the results were inconsistent. In 2002, the mean level for the total EAs was significantly high at one location compared to the others. No comparison was reported in 2003 because ergot sclerotia were only found at one location only due to adverse weather. No significant difference was found between two locations in 2004 (Mainka et al., 2007).

Conversely, Blaney et al. (2009) found that the regions had no effect on the EAs content for ergot-rye sclerotia. Young and Chen (1982) collected grain samples from multiple Canadian fields during the years of 1977 to 1980. The authors found that the ergot sclerotia for triticale and barley contained variable quantities of total EAs. No significant difference was found for the alkaloid composition among the grains collected from the Prairie locations.

McLaren and Flett (1998) explained that cold weather and a decreased in the mean percentage of pollen before the flowering stage were favorable conditions for ergot (*C. africana)*; therefore, these variables can help to predict the ergot's severity in sorghum samples. Also, ergot cannot thrive during mildly hot weather due to an increased in the thickness of the honeydew, which reduces the possibility for the stigma to receive spores; therefore, there are less ergot infections (Miedaner & Geiger, 2015). Menzies et al. (2017) isolated 41 single spores from *C. purpurea* that were collected from different locations in the United Kingdom and Canada. The isolated spores were embedded in different wheat varieties. The authors found that honeydew

production and the weight rate of the sclerotia were higher in the isolates of *C. purpurea* which were collected from the United Kingdom compared to Alberta, Canada, implying that location plays an important role in the ergot's severity based on the *C. purpurea* isolates (Menzies et al., 2017).This experiment showed that location only had a significant effect on the EAs variation in HRS-wheat survey samples during the 2020 growing season only.

Overall, the results for the grading factors in this study revealed an inconsistent interaction effect for the years and the growing locations on the varying test weight. In addition, location had significant effects on the varying test weight and DHV kernels, with the year or across the years. The growing conditions (years and locations) showed no significant influence on the variations for the shrunken and broken kernels, foreign matter, damaged kernels, and total defects.

The results for the non-grading factors suggested an inconsistent interaction effect for the years and the growing locations on the varying moisture content, protein content, and 1,000-kernel weight, except for dockage. In addition, location had significant effects on the variations of the moisture content, protein content, and falling number within the year or across the growing years. Location had significant effects on 1,000-kernel weight within one year. Location only had a significant effect on the DON variation in HRS-wheat samples within the 2019 growing season. Finally, location only had a significant effect on the EAs variation within the year of 2020.

Correlations of Deoxynivalenol and Ergot Alkaloids with the HRS Wheat's Quality Traits

Table 7 shows the correlation of the HRS-wheat quality parameters with DON and the total EAs for all samples obtained from the 2019 and 2020 (combined). DON accumulation in these regions can affect the HRS wheat's quality parameters. McMullen et al. (2012) explained

that FHB can infect kernels and can lower grain yields up to 80%. In addition, FHB can cause damaged kernels and can decrease the test weight, thus affecting the market price and can potentially leading to downgraded grain. In this study, there was an inverse and highly significant relationship (-0.369, P<0.001) between the test weight and the DON content (Table 7). The negative correlation meant that, as the DON content decreases, the test weight tends to increase. Wong et al. (1995) found that a reduced kernel weight was positively associated with *F. culmorum* and *F. graminearum* recoveries. In addition, the current study agreed with Kautzman et al. (2015), who also found that bushel weight was negatively correlated with DON. Quaranta et al. (2010) found an inverse and significant correlation (r= -0.476***) between test weight and DON, and indicated that *Fusarium* caused damaged to the wheat kernels. There was a weak and positive correlation (0.102) between the test weight and the total EAs; however, the relationship was not significant. Dexter and Matsuo (1982) found that the test weight was not influenced by ergot bodies until the ergot level increased to 2%.

Table 7. Simple linear correlation of HRS-wheat quality parameters (2019 and 2020 HRS-samples) with the DON and EAs (n= 207).

Trait [a]	**DON**	**Total EAs**
Grading Factors		
Test Weight	-0.369***	0.102
DHV	-0.129	0.145*
SAB	0.032	0.020
Foreign Matter	-0.067	0.284***
Damaged Kernels	0.324***	0.089
Total Defects	0.257***	0.162*
Non-Grading Factors		
Dockage	0.045	0.138*
Moisture	0.244***	-0.147*
Protein	-0.019	0.255***
Falling Number	-0.166*	-0.029
1,000 -Kernel Weight	-0.090	-0.139*
DON	1.000	-0.054
Total EAs	-0.054	1.000

[a]DHV= Dark, hard, and vitreous kernels; SAB= Shrunken and broken kernels; DON= Deoxynivalenol; EAs= Ergot Alkaloids.

*, **, and *** correspond to $\alpha = 0.05$, 0.01, and 0.001, respectively.

The results in Table 7 revealed a weak and negative correlation (-0.129) between the DHV kernels and the DON content; however, the relationship was not significant. The mean value for the vitreous content reported by Manthey et al. (2004) was 89%. They found an inverse and significant relationship between the DON content and the vitreous content; there was an inverse and significant relationship between the microbial load and vitreous kernels in Durum wheat. Grabowski et al. (2012) found that the mean values for kernel hardness changed during FHB infection. The hardness of a kernel infected with FHB increased slightly during a moderate level of infection, but hardness was reduced during severe infection. There was a positive and significant correlation (0.145, $P<0.05$) between DHV kernels and the total EAs (Table 7). Increased total EAs was correlated with a high percentage of DHV kernels.

There was a positive and weak correlation (0.032) between SAB kernels and DON content; however, the relationship was not significant. This finding concurred with Manthey et al. (2004), who found a weak, positive, and non-significant correlation between SAB kernels and DON, and also found a positive and significant correlation between internal *Fusarium* infection and the SAB kernels. Likewise, EAs showed a weak and positive, but not significant, correlation (0.020) with the SAB kernels. Bechtel et al. (1985) examined the morphological properties of the kernels infected with *F. graminearum*, finding that kernels infected with a high concentration of DON (68.7 ppm) were more damaged and shrunken compared to the less- infected kernels (22.7 ppm), and sound kernels with healthy weight and color had a minimum level of toxins. However, the mean DON values in 2019 and 2020 were 1.5 ppm and 0.6 ppm, respectively, for the current study, which was much lower than what was observed by Bechtel et al. (1985).

There was a weak, inverse, and non-significant correlation (-0.067) between foreign material and DON (Table 7), meaning that increased in foreign matter did not appear to be

associated with the decreased DON content. However, in this experiment most of the mean values for the foreign matter were 0.0%, with varied mean DON values (Table 2). Conversely, EAs presented a positive and highly significant correlation (0.284, $P<0.001$) with foreign material. It is possible that fractions of the ergot sclerotia were presented in the foreign matter even after removing the dockage from the samples.

There was a positive, weak, and highly significant correlation (0.324, $P<0.001$) between the damaged kernels and the DON levels (Table 7). An increased in DON content appeared to be significantly associated with more damaged kernels. However, the EAs presented a positive (0.089) and non-significant correlation with the damaged kernels. Similarly, Manthey et al. (2004) found a positive and significant relationship between the damaged kernels and the DON content ($r= 0.90$) with the Durum wheat samples. Dexter et al. (1996*)* noted that 90% of the damaged kernels were caused by *F. graminearum,* and a strong correlation was found between the DON content and *Fusarium*-damaged kernels with one wheat variety. The study explained that *Fusarium*-damaged kernels deteriorated the baking quality. Kautzman et al. (2015) found a positive and significant correlation ($r= 0.90$) between DON and *Fusarium*-damaged kernels.

The results in Table 7 illustrated the correlations for the total defects in the HRS wheat-samples with DON and EAs. The total defects had a weak, positive, and highly significant correlation (0.257, $P<0.001$) with DON. Likewise, EAs had a positive and significant correlation with the total defects (0.162, $P<0.05$). The total-defects parameter was the only positive and significant correlation that DON and the EAs shared. Therefore, an increased level of both toxins was significantly associated with more total defects. Manthey et al. (2004) found that the total defects had a positive and significant correlation with the DON content ($r= 0.86^{*}$), and suggested that minimal total defects was an indicator for a low microbial load and low DON content; as a

result, total defects can be utilized, along with other grading parameters, as a marker to prevent the purchase of grains with an increased microbial load and high DON levels (Manthey et al., 2004).

The correlation between DON content and dockage was shown in Table 7. There was a positive and non-significant correlation (0.045) between DON and dockage. This result was consistent with Manthey et al. (2014), who found a positive, weak, and non-significant correlation between DON and dockage in Durum wheat. Alhumaid (2016) found a weak, positive, and significant correlation between dockage and DON content when calculating the average dockage for three years (2013-2015) of HRS-wheat samples. Unlike DON, the EAs had a positive, weak, and significant correlation (0.138, $P<0.05$) with dockage (Table 7). This finding illustrated that, as dockage rose, the EAs levels tended to increase. A study by Fajardo et al. (1995) elucidated that the Carter Dockage Tester failed to remove ergot sclerotia due to the similarities between the size of the ergot mass and the healthy seeds. However, in the current study, the relationship between dockage and the total EAs indicated that the ergot mass remained in the sample even though other dockages had been removed.

The results given in Table 7 showed a weak, positive, and highly significant (0.244, $P<0.001$) correlation between the DON concentration and the moisture level in HRS-wheat samples, meaning that, as the moisture content increased, the DON concentration level rose. This finding could be possibly be because the samples harvested during a rainy time had a higher DON content, or because there was high moisture content during storage. Pomeranz (1982) explained that normal kernels have a low respiratory rate with dry conditions. Wheat kernels start to heat when there is increased in moisture content during storage, as a response to a higher respiratory rate. This activity is because of the fungal development (Pomeranz, 1982). The

respiratory rate (production of CO_2) for the wheat and mold increased as the moisture content rose from 12.7% to 19% (Karunakaran, 2001). Surovy et al. (2020) found a tendency of the moisture content to increase slightly when comparing six different categories of damaged wheat that were infected with *Magnaporthe oryzae Triticum* (*MoT*) to the control. However, no significant difference was found for the moisture contents between a healthy wheat (18.36%) compared to a severely infected wheat with *MoT* (18.73%; more than 80% damage). Birzele et al. (2000), found that keeping wheat with 17% and 20% moisture content at 20°C, over 6 weeks, resulted in an increased DON content. When storing the 1997 wheat samples with 17% moisture content, the DON level rose 9 fold in four weeks, and the DON level rose five fold in six weeks with the 1998 samples. The study also indicated that the increased DON content during poor storage was based on the *Fusarium* species that aggressively produced DON, rather than the initial increase with the DON level and FHB.

Although there was a positive and highly significant relationship between DON and the moisture content, there was an inverse and significant correlation (-0.147, $P<0.05$) between moisture content and the EAs level. As the moisture content increased, the EAs level decreased. No available research explained this relationship. However, Coufal-Majewski et al. (2016) explained that quickly drying the cereals and proper storage are important to prevent a higher moisture content, which increases the chance of having alkaloids present.

There was no significant correlation (-0.019) between the protein content and the DON content (Table 7). This finding suggested that increased protein content had no influence on reducing the DON content. This weak relationship is probably because the total means for the DON contents in 2019 and 2020 were less than 2.0 ppm. Therefore, the protein content was not affected by the DON level. A study found that a low protein content was associated with

increased DON content and explained that rainy weather and untimely harvesting can increase the chance of mycotoxin contamination, thus affecting wheat's quality (Kochiieru et al., 2021). Gärtner et al. (2008) observed a slight reduction for the protein contents in different HRS-wheat cultivars that were infected with *Fusarium*, compared to the control, yet the protein contents were still between 13% and 15%. However, other studies found different results. Quaranta et al. (2010) found a positive and significant correlation between DON and protein content ($r = 0.179^{**}$) for Durum wheat. Hamilton and Trenholm (1984) found increased protein content for wheat that was contaminated with DON. They explained that the increase might be coming from the fungus' hyphae, which consists of approximately 42% protein, or because the *Fusarium* consumed the sugar and starch during the development. Siuda et al. (2010) observed an increased protein content for wheat infected with *Fusarium* when the degree of infection was more than 10%. Boyacioğlu and Hettiarachchy (1995) studied wheat infected with *F. graminearum*; they found an increase in the protein content for the moderately infected wheat (16.8%) compared to the control and lightly infected wheat (15.8% and 15.6%, respectively). On the other hand, the EAs showed a positive and highly significant ($P<0.001,0.255$) correlation with the protein content. Dexter and Matsuo (1982) found a higher level of protein as the percentage of the added ergot sclerotia in the wheat increased. Pomeranz et al. (1975) found that healthy kernels contained less protein compared to the amount found in the ergot sclerotia of *C. purpurea*, but there was a difference in the protein compounds found in the ergot sclerotia compared to the healthy kernels.

There was an inverse and significant ($P<0.05$) correlation (-0.166) between the falling number and the DON content (Table 7). This finding suggested that a high falling number was significantly associated with a low DON level. Similarly, the EAs showed an inverse correlation

(r= -0.029), yet the relationship was non-significant (Table 7). Kochiieru et al. (2021) found a negative and significant correlation between the DON content and the falling number in Spring wheat samples that were harvested in 2016, 2017, and 2018 (r = -0.95, r = -0.89, and r = -0.46, respectively) and concluded that rainy weather and an untimely harvest lowered the falling number. For example, as the grain reached full maturity with the 2016 samples, the falling number of the harvested samples contaminated with one mycotoxin was within the acceptable falling-number value (399 sec). However, harvesting after 12 days and 20 days of full maturity resulted in a significant reduction for the falling number (Kochiieru et al., 2021).

Thousand-kernel weight showed an inverse and low correlation with DON, indicating that an increased TKW had no pronounced association with the reduction of DON content (Table 7). This correlation agreed with Chełkowski et al. (2000). The correlation between TKW and the DON level was negative and non-significant (r= -0.027) for the barley samples inoculated with *F. culmorum*. The ergot alkaloids had a significant (-0.139, P<0.05) and inverse relationship with TKW. Quaranta et al. (2010) found an inverse and significant correlation (r= -0.243***) between TKW and DON; they explained that the reduced TKW was possibly due to the fungi damaging to the seed. Surovy et al. (2020) found a significant decrease for the TKW in wheat infected with *MoT*. The minimum value of the TKW found in wheat samples infected with *MoT* was 17.88 g, with 80%-99% damaged kernels, compared to the maximum value of 42.7 g in healthy wheat kernels (control).

Table 7 presents a negative and non-significant correlation (-0.054) that appeared between DON and the EAs contents. This finding suggested that an increased DON content had no significant relationship with the decreased EAs or vice versa. This weak relationship was observed in a study by Schwarz et al. (2006) when they examined 304 barley samples from three

states in the U.S. Midwest. The authors found a correlation coefficient of *r*= 042, with no obvious relationship between the ergot sclerotia and the DON content, although the majority of those ergot samples (>90%) contained DON, with concentrations that ranged from 0.1 μg/g to 69 μg/g. However, in the current study, the highest DON level was found in MN region B (4.5 ppm) for the 2019 growing season, and the mean EAs concentration in that area was 69.3 ppb (Table 2). Other areas, such as SD region B in the 2020 growing season, had the highest amount of EAs (374.4 ppb), and the DON level was 0.0 ppm (Table 2). This inverse relationship between the production of DON and EAs in the HRS-wheat survey samples, could be due to the nature of the sample as well as some environmental or biological factors.

Scanning Electron Microscope Images for Ergot Sclerotia of *C. pupurea*

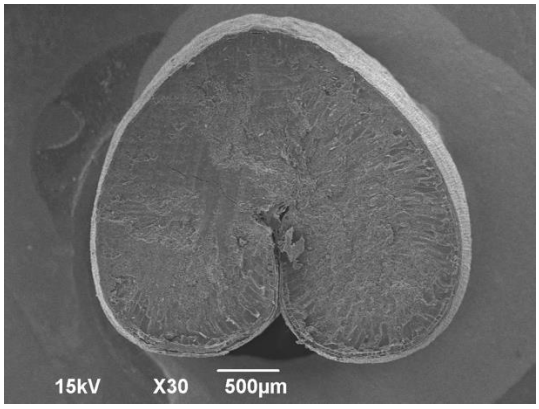
Cross-section images of a healthy kernel compared to ergot sclerotia samples are illustrated in Figures 5, 6, and 7. The healthy, cleaved kernel shows the whole kernel, the endosperm, and the bran layers. The images of the ergot sclerotia separated from the CMS-HRS wheat present the whole mass, the center, and the edge. In the healthy kernel, the part from the outer pericarp to the crease had a visible center cavity and pigment strand, but they were absent in the ergot sclerotia of the CMS-HRS wheat in all treatments except Priaxor's sample.

For the non-treated control sample (Figure 5), the image shows the whole transversally cleaved ergot sclerotium magnified at 25x. The ergot mass appeared with a deformed crease. The outer pericarp layer looked cracked and uneven. The Miravis Ace ergot sample also showed similar characteristics. The outer pericarp was wrinkled compared to the healthy sample, and the crease was widely opened and contained a crack in the middle. Likewise, the sclerotium sample treated with Quilt revealed a deformed crease and contained a few cracks in the pericarp. In contrast, the sclerotium sample treated with Priaxor looked less affected than the other treatments

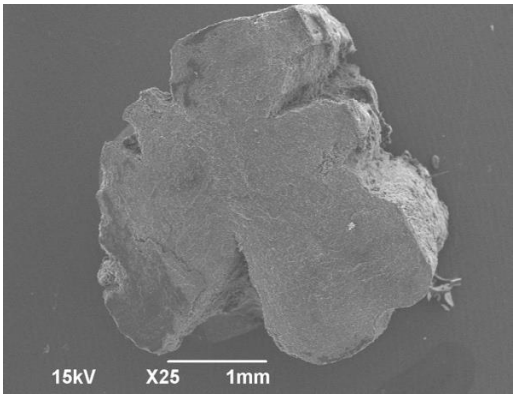
but still not perfectly shaped when compared to the healthy kernel. In the center cavity area of the Priaxor sample, there was a cluster of branches surrounded by very fine threads. Last, the sample for the Sphaerex fungicide appeared flat and showed no crease with a few cracks around the outer part but not as deep compared to the other samples.

Figure 6 shows an inner endosperm of the healthy kernel and the inner part for the ergot sclerotia. Healthy endosperm was described by Pomeranz (1982) who mentioned that the wheat kernels' inner endosperm contains small and large starch granules that are tightly packed and that protein matrices are distributed around the granules. In wheat, the large type (A) granules are disk-shaped, while the small type (B) granules are spherical-shaped (Lin Jane, 1996). In contrast to the healthy kernel, the inner part of the ergot body for the non-treated control, when enlarged at1,000x, revealed that the granules were possibly consumed by the fungus and might be replaced with aggregated branches of hardened mycelium that have different pore sizes.

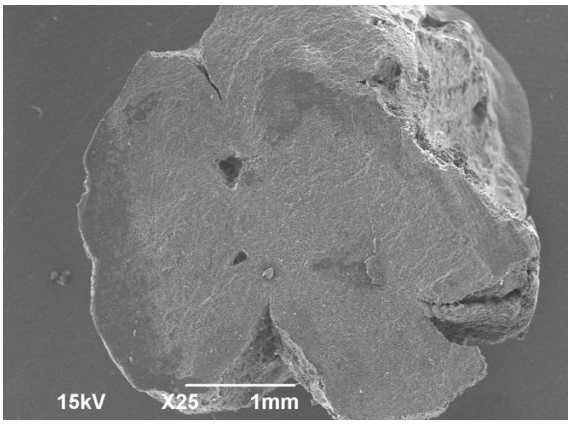
According to Lorenze and Hoseney (1979), the mycelium filled out the entire kernel's tissue and replaced the grain composition. A scanning electron microscope was previously utilized to examine ergot sclerotia of triticale by Lorenze and Hoseney (1979), and they described the ergot sclerotia's center structure as a "highly irregular and porous structure of intertwined hyphae of the mycelium" (Lorenze & Hoseney, 1979, p.312). They also explained, that the inner structure of the ergot body in wheat is different from rye where the hypha of the ergot body tends to be less "fissured."



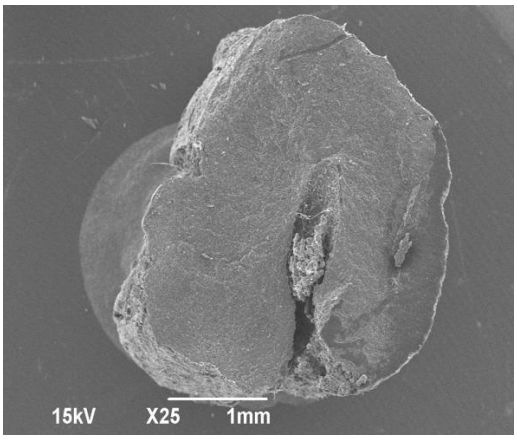
Healthy kernel HRS wheat



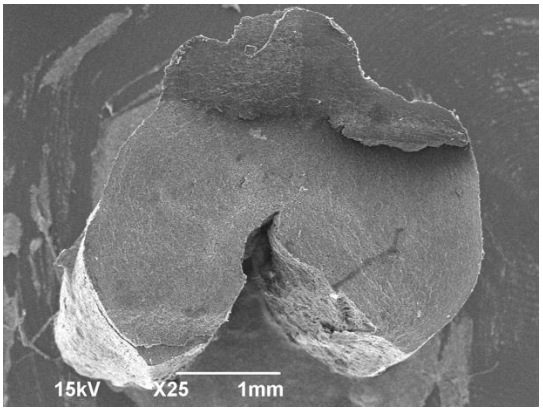
Non-treated control



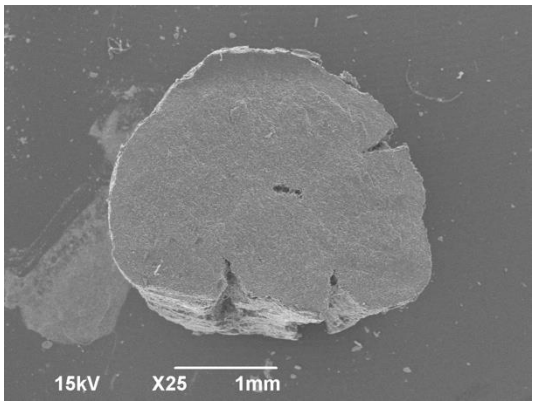
Quilt



Priaxor



Miravis Ace



Sphaerex

Figure 5. Microscopic images of a transversally cleaved, healthy kernel and ergot sclerotia (*C. purpurea*) from wheat treated with different fungicides, and non-treated control sample.

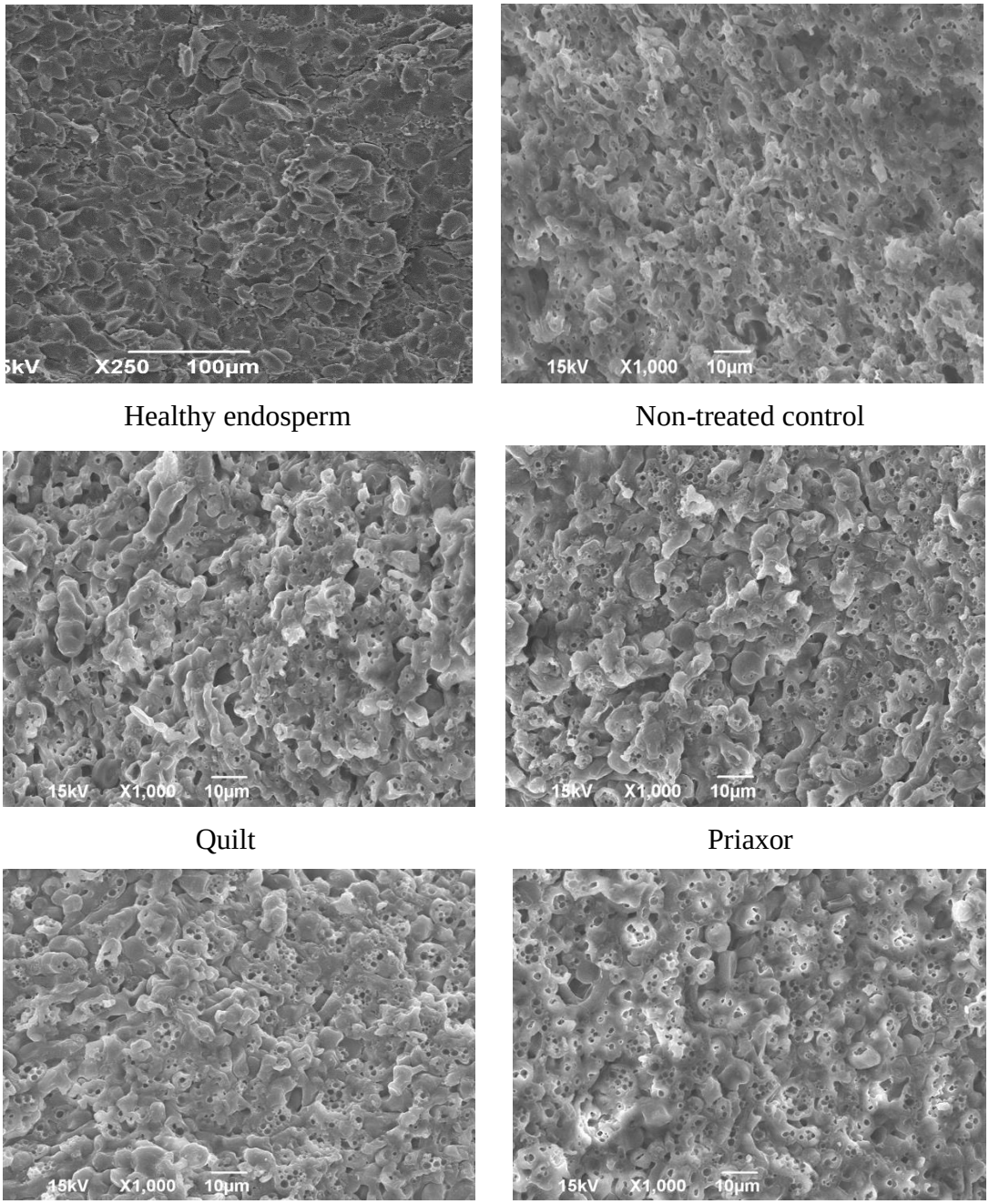
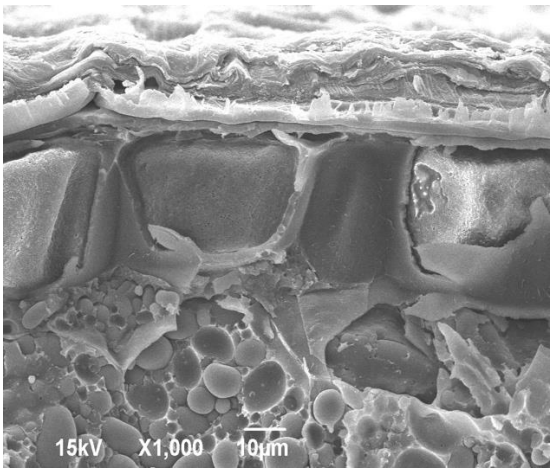
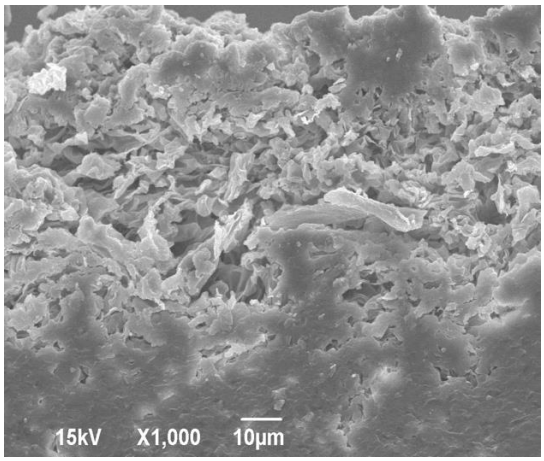


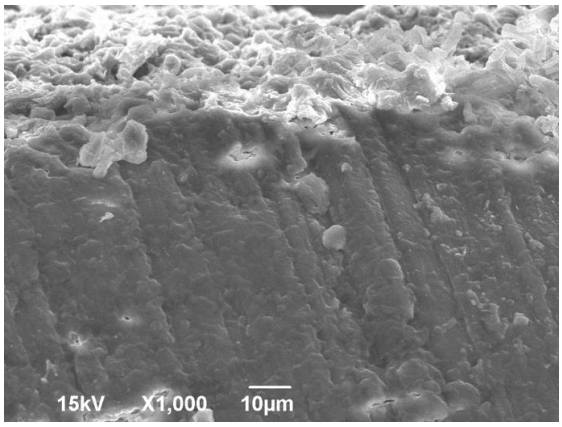
Figure 6. The center of a healthy kernel and ergot sclerotia (*C. purpurea*) from wheat treated with different fungicides, and non-treated control sample.



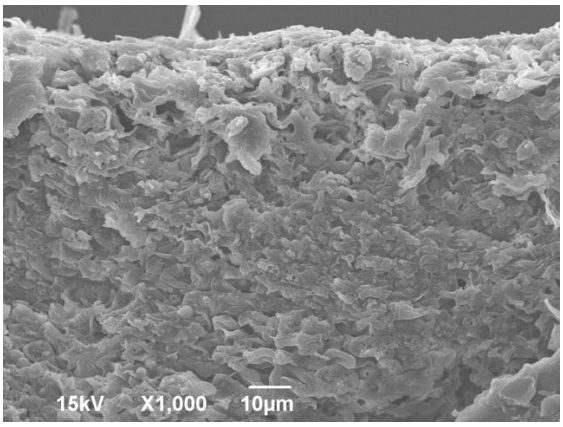
Healthy Kernel



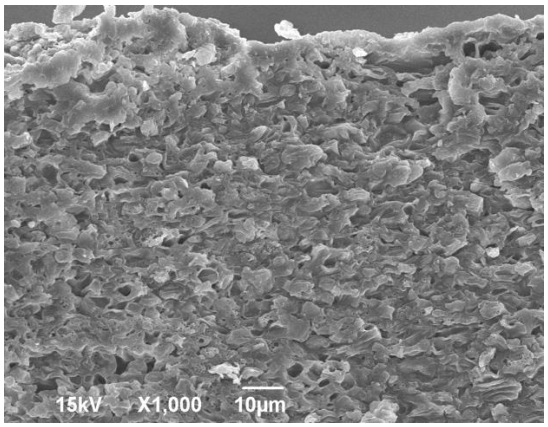
Non-treated control



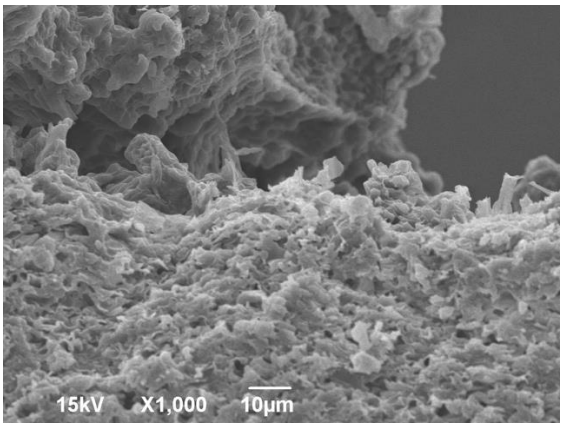
Quilt



Priaxor



Miravis Ace



Sphaerex

Figure 7. Microscopic images of the bran layer of a healthy kernel and the edges of the ergot sclerotia (*C.purpurea*) from wheat treated with different fungicides, and non-treated control sample.

Schumann and Uppala (2000) mentioned that the sclerotia's mycelium is white in color and contains storage cells. The mycelium contains a protective, dark-colored layer, known as the outer cortex, which provides the mycelia with protection from dryness (Schumann & Uppala, 2000). The center part of the ergot sclerotium treated with Miravis Ace showed the absence of the starch granules and had a deformed shape, compared to the normal inner structure of a healthy kernel. The sclerotium treated with Miravis Ace shared characteristics with the non-treated control sample, but with more pitting on these branches (hyphae). In addition, the domestic structure of the ergot sclerotia treated with the Quilt, Priaxor, and Sphaerex fungicides also possessed asymmetrical branches with different sizes of small holes, and none of the inner parts of these sclerotia were close to the starch granules of the healthy kernel. Young (1981) reported that the EAs were concentrated in the center of the rye sclerotium, while Silber and Bischoff reported that the outer layers of the ergot mass contained more alkaloids compared to the center (Young, 1981). The sclerotium's inner structure was rich in fat and had some nutrients that help with the germination or the fruiting stage (Alderman, 2006).

The healthy kernel's bran layer is shown in Figure 7; there are noticeable aleuron cells and a pericarp. Aleuron cells, the pericarp, and seed coats are the structure of the bran. The aleurone layer is high in protein and amino acids, and it is the outer part of the endosperm, which is characterized by large cells that are cubic in shape (Pomeranz ,1982). The image illustrates the non-treated control sclerotium, and it appears with misshaped aleuron cells and a different pericarp compared to the healthy kernel. The Miravis Ace, Priaxor, and Quilt samples show the edges of ergot bodies, and the edges appear to be more compact compared to the non-treated control sclerotium but are still without aleuron cells. The edges of the ergot sclerotium treated with Sphaerex also appear to be damaged with no visible aleuron cells. Alderman's (2006) cross-

sectional image of *C. pupurea* illustrates that the ergot sclerotium's outer layer, or the "rind," is essential to protect the ergot body from the outer environment, such as insects (Alderman, 2006). More research is needed to quantify the ergot mycelia's weight in order to examine the difference between these fungicides.

Variations of the Ergot Sclerotia's Physical Parameters Among the Treatments

In Table 8, the mean values for total plot weight of the clean and unclean (combined) samples, which were collected from the four replicated plots, with each treatment revealed that the mean value of the total plot weight of the non-treated control samples was 238.75 g, and the highest and lowest mean values for the total plot weight were found for Priaxor (284.50 g) samples, and Miravis Ace (217.75 g) samples, respectively. In this study, the term "ergot body weight" refers to the total weight of ergot sclerotia from each plot, after separating the ergot sclerotia from other contents. The mean value for the ergot body weight in the non-treated control samples was 70.17 g while the lowest ergot body weight was found with Miravis Ace, 39.57 g samples.

Miravis Ace treated samples presented slightly high mean values for the length and width of ergot sclerotia: 7.99 mm and 3.45 mm, respectively. The lowest mean value for the length of ergot sclerotia was found with Quilt treated samples, 7.44 mm, and the lowest mean value for width was found with Priaxor treated samples, 3.17 mm.

The ergot size varied among ergot species, and in general, the length was between 2 and 50 mm, and the diameter is relatively small (measured in millimeter; Tenberge,1999). The length varied for other ergot species, ranging from 8 cm long in *C. gigantea* to 3 mm long in *C. microcephala* (Lorenz & Hoseney 1979). In addition, the size of ergot sclerotium is varied among host plant (EFSA, 2012). In the current study, the mean value of the individual ergot

weight was uniformed for all treatments at 0.03 g. The average weight of the ergot sclerotia for rye samples, according to Blaney et al. (2009) and collected in 2008, was from 0.2-3.0 g.

Table 8. Mean and least significant difference for the ergot-sclerotia's parameters Among Fungicides (n= 20).

Treatment	TPW (g)	EBW (g)	LTH mm	WTH mm	WGT (g)	MC %	ASH% (14% MB)	PC % (14% MB)	EAs ppb	DON ppm	NIV ppm	FUX ppm	3ADON ppm	15ADON ppm	DAS ppm	ENNS ppm	HT2 ppm	T-2 ppm	ZEN[a] ppm
Non-treated	238.75	70.17	7.87	3.18	0.03	6.95	3.36	29.32	254,330	0.24	0.02	0.00	0.10	0.02	0.04	0.03	0.13	0.03	0.11
Quilt	250.00	55.83	7.44	3.27	0.03	7.08	3.39	29.03	352,375	0.17	0.03	0.00	0.09	0.02	0.03	0.03	0.24	0.03	0.09
Priaxor	284.50	55.04	7.97	3.17	0.03	7.00	3.35	29.46	244,840	0.21	0.04	0.01	0.12	0.01	0.05	0.06	0.16	0.05	0.05
Sphaerex	233.25	54.82	7.73	3.38	0.03	6.97	3.29	29.27	359,485	0.14	0.02	0.00	0.16	0.02	0.02	0.05	0.30	0.04	0.07
Miravis Ace	217.75	39.57	7.99	3.45	0.03	6.97	3.59	28.72	339,260	0.07	0.01	0.00	0.14	0.01	0.04	0.04	0.12	0.04	0.09
LSD (p=0.05)[b]	**NS**	**9.81**	**NS**	**NS**	**NS**	**NS**	**0.29**	**NS**	**105,947**	**0.11**	**NS**	**NS**	**NS**	**NS**	**NS**	**NS**	**NS**	**NS**	**NS**
CV%[c]	**37.2**	**11.6**	**8.8**	**12.0**	**20.1**	**2.6**	**5.5**	**1.9**	**22.2**	**41.9**	**108.8**	**74.6**	**67.0**	**47.1**	**45.2**	**93.8**	**89.0**	**61.5**	**82.2**

[a]TPW= Total plot weigh, EBW= Ergot body weight, LTH= Length of ergot sclerotium, WTH=Width of ergot sclerotium, WGT= Weight of ergot sclerotium, MC= Moisture content, ASH= Ash (14 % moisture basis), PC= Protein content (14 % moisture basis), EAs= Total ergot alkaloids, DON= Deoxynivalenol, NIV= Nivalenol, FUX= Fusarenon-x, 3ADON= 3-acetyl deoxynivalenol, 15ADON= 15-acetyl deoxynivalenol, DAS= Diacetoxyscirpenol, ENNS= Neosolaniol, and ZEN= Zearalenone.

[b]LSD = Least significant difference.

[c] CV= Coefficient of variance.

In Table 9, the ANOVA shows the effect that fungicides have on the parameters of the ergot sclerotia. The results illustrate that there were no significant (P>0.05) variations found with the total plot weight, width, length, and weight of ergot sclerotium. This indicates that the treatments had no significant influence on the variances of these four parameters. The only significant (P<0.05) variation was found with the ergot body weight, which means that the treatments had an effect on the ergot body weights' variance.

Table 9. F-values for the effect of treatments on ergot-sclerotia's parameters (degrees of freedom= 4).

Parameters [a]	Mean Square	F-Value	Pr> F[b]
TPW	2504.8	0.30	0.8712
EBW	469.0	11.56	0.0004
LTH	0.20	0.44	0.7793
WTH	0.1	0.40	0.8062
WGT	0.0	0.52	0.7221
Moisture content	0.0	0.34	0.8482
Ash content	0.1	1.51	0.2599
Protein content	0.3	1.07	0.4123
EAs	1.24E+10	2.63	0.0869
DON	0.0	3.79	0.0323
NIV	0.0	0.66	0.6319
FUX	0.0	0.61	0.6621
3-ADON	0.0	0.47	0.7579
15-ADON	0.0	0.72	0.5937
DAS	0.0	0.97	0.46
ENNS	0.0	0.44	0.7747
HT-2	0.0	0.85	0.5181
T-2	0.0	0.24	0.9079
ZEN	0.0	0.54	0.7076

[a]TPW= Total plot weight, EBW= Ergot body weight, LTH= Length of ergot sclerotium, WTH=Width of ergot sclerotium, WGT= Weight of ergot sclerotium, EAs= Total ergot alkaloids, DON= Deoxynivalenol, NIV= Nivalenol, FUX= Fusarenon-x, 3-ADON= 3-acetyl deoxynivalenol,15-ADON= 15- acetyl deoxynivalenol, DAS= Diacetoxyscirpenol, ENNS= Neosolaniol, and ZEN= Zearalenone.
[b]Pr>F= probability.

In Table 8, the LSD, at P= 0.05, was also conducted to determine the mean significant difference among the treatments for each parameter. No significant (P>0.05) differences were

found among the mean values of all the treatments for the total plot weight, length, width, and weight of the weight of ergot sclerotium. However, all fungicides had a significantly lower ergot body weight compared to the non-treated control samples. In addition, the mean value of the ergot body weight with Miravis Ace was significantly less (39.57 g) compared to the ergot body weight with Quilt (55.83 g), Priaxor (55.04 g), and Sphaerex (54.82 g).

Research regarding the effect of fungicides on *C. purpurea* was very limited; however, some these studies evaluated the ergot body's reduction or other parameters after fungicides' application. Kaur et al. (2018) tested the efficacy of five fungicides on perennial ryegrass infected with *C. purpurea*: Priaxor (FRAC codes 7+11: fluxapyroxad + pyraclostrobin), Solatenol (FRAC code 7, benzovindiflupyr), Fontelis (FRAC code 7: penthiopyrad), Propulse (FRAC codes 3+7, prothioconazole + fluopyram), and Quilt Xcel (FRAC codes 3+11, propiconazole+ azoxystrobin). The fungicides were applied three times during the time of anthesis, starting at Feekes stage 10.51. The researchers found that Priaxor, Propulse, and Quilt significantly reduced the production of honeydew. Also, all these treatments significantly reduced the disease severity when compared to the control. The study indicated that Quilt was the "industry standard" for grass seed, and some of these fungicides showed comparable efficacy to control ergot.

The Quilt fungicide was discussed by Wu et al. (n.d.) to test its efficacy on *C. purpurea* infection and ascospores production in Kentucky bluegrass. Quilt was applied at different times during the flowering stage. In one field, the ergot sclerotia's incidence in a plot treated with Quilt was not significantly different from the non-treated control plot when the fungicide was applied once on June 8. Also, ergot sclerotia's incidence on June 8 was significantly higher compared to the plot where fungicide was applied once on June 15 and the plot were the fungicide applied

twice (June8 and 15). In the other field, Quilt with different application times (June 8 or 15 or both) reduced ergot sclerotia's incidence, but did not create significantly different results.

Walenta et al. (2009) also used Quilt in their study and found that the percentage of ergot *C. purpurea* infection was absent (0%) in all samples of Kentucky bluegrass treated with Quilt. The seed yield was higher when the fungicide was applied twice (at early heading and early anthesis) than when the same fungicide with a single or three applications, which can save up to $60/acre. The authors also found that a low number of ascospores was relative to a low level of ergot infection. In addition, neither temperature nor soil moisture affected the release of ascospores.

Dung et al. (2018a) observed the effect of the Priaxor SC, Quilt Xcel SE, A19649B (FRAC code 7), Aproach 2.08 SC (FRAC code 11), Trivapro SE (FRAC codes 3+7+11) fungicides on two Kentucky bluegrass varieties at the flowering stage. Then, from each type, 100 seed heads were collected, and the number of sclerotia from each panicle was determined to quantify the ergot's severity. The percentage of panicles was calculated to quantity the incidence of sclerotia. All the fungicides with one variety, except one fungicide, significantly decreased the ergot's severity and incidence compared to the control. Also, these fungicides were not statistically different than the standard Quilt Xcel for grass. In the other variety, all fungicides significantly decreased the ergot's severity and incidence compared to the control.

In the current study, all fungicides were applied once at Feeks 10.5 (full-head stage) in the plots before anthesis. The mean values for the ergot body weight (g) samples were significantly less with all fungicides compared to the non-treated control. In addition, utilizing Miravis Ace led to a significantly smaller mean value for the ergot body weight (39.57 g) when compared using to Quilt (55.83 g), Priaxor (55.04 g), or Sphaerex (54.82 g, Table 8).

The fungicides' phytomobility refers to their movement inside the plant tissue (Mueller et al., 2020b). In this study, all fungicides were systematically absorbed by the plant and classified as acropetally mobile, which means that they move upward within the xylem vessel of the plant to the leaf (Mueller et al., 2020b). The lipophilicity (logP), refers to the capability of the active ingredient to dissolve in solvents (e.g., oil, fat, or lipids), and the translocation stream concentration factor for each active ingredient in these fungicides were other factors associated with these fungicides' bioavailability (Mueller et al., 2020b). However, these factors were unlikely to be the reasons for these differences among the fungicides; nevertheless, the primary reason for these differences could be related to the mode of action among the active ingredients. Quilt and Miravis Ace contain propiconazole (FRAC code 3), but the active ingredient pydiflumetofen (FRAC code7) presented in Miravis Ace might be more efficient for reducing the ergot body weight compared to Quilt or other fungicides (Table 8). The carboxamides under FRAC code 7 trigger Complex II in fungal respiration (FRAC, 2005), and can disturb the mitochondria's TCA cycle by inhibiting the SDH enzyme (Scalliet et al., 2012).

Varying the Moisture, Protein, and Ash Contents of Ergot Sclerotia Among the Treatments

The mean value for the moisture content of the ergot sclerotia was slightly high in the samples treated with Quilt (7.08 %) compared to the other fungicides, and the lowest mean value for the moisture content was found in the non-treated control samples (6.95%). In addition, ergot sclerotia of the Priaxor fungicide exhibited a slightly higher mean value for the protein content (29.46 %), and the lowest mean value was observed with the Miravis Ace treated samples (28.72 %). The mean value of the ash content was 3.59 % in the Miravis Ace treated samples, which was slightly higher compared to the other treatments, and the lowest mean value was found with the Sphaerex treated samples (3.29%, Table 8).

The ANOVA in Table 9 presents no significant (P>0.05) variations for the moisture, ash, and protein contents, meaning that the treatments had no significant effect on these parameters' variances. The LSD test in Table 8 showed no significant (P>0.05) differences among the mean values of all treatments for the moisture and protein contents, except for ash. The results indicated that the mean value for the ash content of the treated samples with Miravis Ace (3.59%) was significantly (P<0.05) higher compared to the mean value of the treated samples with Sphaerex (3.29%).

The current study showed very low mean values for the moisture content that ranged from 6.95% to 7.08%.There was no study available to explain the moisture content in relation to ergot sclerotia, but according to Grabowski et al. (2012), the individual kernel infected with FHB had a lower moisture content due to the utilization of the fungi to the moisture during growth.

Regarding the protein content, Dexter and Matsuo (1982) studied the effect of contaminating Durum wheat with ergot sclerotia of *C. purpurea*. They found that an increased severity for the ergot level was associated with higher protein levels that ranged from the lowest (12.8%) in the control to 13.4% in the affected kernels (when the ergot body was added at 5.0%). Ash content was influenced when the ergot level was above 2.0%, and the ash content was 1.51% in the control and 1.58% in the in the affected Durum-wheat samples (when the ergot body was added at 5.0%). However, the current study examined the ash content of ergot sclerotia samples not containing any wheat kernels, and showed a higher level of ash contents with all treatments. Lorenze and Hoseney (1979) indicated that ergot sclerotia's ash content was between 2.2 and 6.5%, which is relative to the ash content reported in this study (mean values between 3.29 and 3.59%). Lorenze and Hoseney (1979) added that, among the 27 minerals found in ergot sclerotia, phosphorus was the most important element for the maturation of ergot sclerotia.

Pomeranz et al. (1975) found that healthy kernels contained less protein compared to the protein found in the ergot sclerotia of *C. purpurea*, with differences in the protein compounds that were found in the healthy kernels and the ergot sclerotia.

This study measured the protein, ash, and moisture contents presented in the ergot sclerotia that separated from the CMS-HRS wheat. There was no direct comparison with other studies; however, a similar trend, with different fungi, was found in other studies. Boyacioğlu and Hettiarachchy (1995) performed a study on infected wheat with *F. graminearum*. They found increased protein content for the moderately infected wheat (16.8%) compared to the control and lightly infected wheat (15.8% and 15.6%), respectively. They explained that the increased in the protein content might be coming from the hyphae of the fungus, which consists of 42% protein. Another study by Surovy et al. (2020) indicated that protein contents were increasing with the severity of *Magnaporthe oryzae Triticum* (*MoT*) infection. The protein content's range was from 9.69% to 18.53%. The ash content was also elevated with the increased disease severity, ranging from 1.99% to 2.35%; however, the authors did not find a clear explanation for the increased ash (Surovy et al., 2020). The current study showed that ergot sclerotia of the CMS-HRS wheat, had low moisture contents, had very high protein contents, and had high ash contents.

Varying the Saprophytic *Fusarium* Toxins in Ergot Sclerotia Among the Treatments

The samples that were evaluated for *C. purpurea* were also naturally infected with *Fusarium*. The saprophytic *Fusarium* colonized ergot sclerotia and produced toxins, which was evident by the presence of DON, trichothecenes groups A and B, and ZEN during the toxin analysis (Table 8). A very slight variation was observed with the NIV; the lowest mean level was observed for the treated samples with Miravis Ace (0.01 ppm), and the highest mean value was

found in the treated samples with Priaxor (0.04 ppm). Fusarenon-x (FUX) was absent in all treated samples except Priaxor (0.01ppm). Also, the lowest mean amount of 3-ADON was found in Quilt treated samples (0.09 ppm), but was high when utilizing Sphaerex (0.16 ppm). The lowest mean value of 15-ADON was found with the Miravis Ace and Priaxor treated samples, and the measurements were similar at 0.01 ppm (Table 8).

The lowest mean level of Diacetoxyscirpenol (DAS) was found with Sphaerex treated samples (0.02 ppm), and Priaxor treated samples presented a slightly high mean value (0.05 ppm). Neosolaniol (ENNS) levels were less in the non-treated control and Quilt samples, and they were similar at 0.03 ppm; the ENNS level was slightly high in Priaxor treated samples, with a mean value of 0.06 ppm. The lowest mean value of H2-T toxin was found in Miravis Ace treated samples (0.12 ppm); there was a slight elevation in Sphaerex treated samples (0.30 ppm).The lowest mean amount for the T-2 levels was found with the non-treated control and Quilt (0.03ppm) samples, and the mean level was slightly higher with Priaxor (0.05 ppm) samples. Finally, the mean value for the ZEN level was high in the non-treated control samples (0.11ppm), and the lowest amount was found in the Priaxor treated samples that had a mean value of 0.05 ppm (Table 8).

The ANOVA indicated that fungicide treatments had a significant ($P<0.05$) effect on the variation for DON content (Table 9). Additionally, fungicide treatments had no significant ($P>0.05$) effect on the variations for NIV, FUX, 3-ADON, 15- ADON, DAS, ENNS, HT-2, T-2, and ZEN (Table 9).

The LSD test in Table 8 revealed that Quilt, Priaxor, and Sphaerex treated samples had mean DON-content values that were less compared to the mean value of the non-treated control, but the difference was not significant ($P>0.05$). The results demonstrated that the mean value of

the DON level when using Miravis Ace (0.07 ppm) was significantly ($P \leq 0.05$) less than the mean value of the non-treated control and the Priaxor treated sample (0.24 and 0.21ppm, respectively). In addition, no significant ($P > 0.05$) differences were found among the mean values of the all fungicides regarding the levels of NIV, FUX, 3-ADON, 15-ADON, DAS, ENNS, HT-2, T-2, and ZEN (Table 8).

According to field observations at the NDSU Agricultural Experiment Station-Fargo, *Fusarium* mycelia were presented on the ergot sclerotia. The natural saprophytic *Fusarium* fungi colonized the ergot body, which possibly means that the ergot infection commenced prior to the saprophytic *Fusarium* infection. According to Kazan and Gardiner (2018), *F. graminearum* is known as a saprophytic fungus, therefore, it can colonize a host's dead tissue during necrosis development (Kazan & Gardiner, 2018). *F. graminearum* was found to act as a pathogenic fungus on living wheat heads and produced a high level of mycotoxins, but it produced fewer toxins on the wheat heads' dead tissue during the saprophytic phase (Boedi et al., 2016). In general, the results showed very low levels of trichothecenes and ZEN toxins measured in the ergot sclerotia, with a very slight variation for the mean values among the fungicide treatments.

In the recent study, although, Sphaerex contains two active ingredients of triazoles while Miravis Ace contains a triazole (propiconazole) and another active ingredient (Table 1), Miravis Ace performed better than Sphaerex and had samples with a significantly lower DON level compared to the control. This distinction could possibly be due to the difference with the active ingredients' efficacy. Applying Miravis Ace prior to anthesis at Feeks 10.5 in this study was effective in reducing the DON content, and Singh et al. (2021) found a similar trend when they used Miravis Ace to control FHB in wheat, but their application was at a different Feeks. Singh et al. (2021) discovered that applying the fungicide before anthesis at 50% head emergence

(Feeks 10.3) was significantly effective for reducing the DON content by 52%, and the disease severity by 69% compared to the control. They also found that utilizing Miravis Ace at anthesis significantly reduced the DON content by 54.89% and the disease severity by 66.94% when compared to the control.

Also, many prior studies reported that triazoles effectively reduced the DON content. Beyer et al.'s (2006) review mentioned that the mean efficacies of triazoles on the DON level in 10 field studies were between 10 and 67%, and the triazoles' efficacy was based on the time of the fungicide application (Beyer et al., 2006). No published study examined the effect of Sphaerex on the DON content in wheat. Sphaerex contains triazoles, and there was a low level of DON content in the current study compared to the non-treated control, but the difference was not significant.

Paul et al. (2018) described how the combination of QoI and DMI (azoxystrobin and propiconazole) at Feeks 10.5, which corresponded to Quilt in this study, elevated the DON content in wheat, but other combinations did not elevate the DON concentration. Therefore, the effect of the DMI and QoI combination on the DON level was based on the mixture's active ingredients. Paul et al. (2018) mentioned that a more efficient DMI, for instance prothioconazole or metconazole, can diminish the QoI's adverse effect on DON level.

This study did not observe an increase of DON contents when Quilt or Priaxor were applied before anthesis compared to the non-treated control samples, but the DON level did not show a great response, especially with the Priaxor fungicide. Although the DON content with Priaxor (0.21ppm) was less than the non-treated control (0.24 ppm) in the current study, the DON level was significantly higher than the sample with Miravis Ace. The Quilt treatment (0.17 ppm) was also less effective after Priaxor, but there was not a significant difference.

Marques et al. (2017) observed the effect of several fungicides on three wheat types; and one fungicide was fluxapyroxad + pyraclostrobin, which correspond to Priaxor in this study. Marques et al. (2017) found that this fungicide combination controlled FHB in the plot, but had a poor effect with reducing the DON content, which was consistent with this study. The authors also implied that, when triazole is absent in the fungicides, it will be difficult to control DON.

Varying the Ergot-Alkaloid Content Among Treatments

The mean values for the total EAs of ergot sclerotia revealed that the highest mean content of total EAs was found in Sphaerex treated samples with a mean value of 359,485ppb, and the lowest mean concentration of total EAs was found in Priaxor treated (244,840 ppb) samples. Also, the mean values for the total EAs in Sphaerex, Miravis Ace, and Quilt treated samples were higher than the non-treated control (Table 8). In addition, the ANOVA in Table 9 indicated that the treatments had no significant ($P>0.05$) effect on the variation of total EAs. The LSD in Table 8 revealed that the mean value for the total EAs in the Priaxor treated samples was 244,840 ppb, which was significantly ($P\leq 0.05$) less than the mean values found in Sphaerex and Quilt treated samples (359,485 ppb and 352,375 ppb, respectively). No significant ($P>0.05$) difference was found between Priaxor and the non-treated control (254,330 ppb) or Priaxor and Miravis Ace (339,260 ppb) treated samples.

Regarding the level of total EAs, no prior studies conducted a toxin analysis of the EAs after fungicides application. This study observed an increased in the level of EAs when using Sphaerex, Quilt, and Miravis Ace, compared to the non-treated control, but the difference was not significant (Table 8). There was no previous research that was done to explain this phenomenon on total EAs. As explained earlier, these three fungicides created a significantly low ergot body weight (g) compared to the non-treated control.

In this study, a single application of Priaxor had more a profound effect on the level of EAs compared to the other treatments. Specifically, Priaxor treated samples was significantly less in the total EAs than Sphaerex and Quilt treated samples, and less than the non-treated control (but not significantly). This reduction in EAs level, could be related to Priaxor's active ingredients are fluxapyroxad (SDHI) and pyraclostrobin (QoI). According to Duvenage et al. (2019), SDHIs are used with plant disease due to their ability to attach to the ubiquinone binding (Q_p) site (Duvenage et al., 2019). Changes in the SDH enzyme cause greater amount of succinate and higher oxygen toxicity (Moreno et al., 2020). In the inner membrane of the mitochondria, the catalytic reaction of Complex III leads to the movement of electrons from ubiquinol, which attach a Q_o site, and eventually to cytochrome c (Meunier et al., 2013). However, Bartlett et al. (2002) explained that QoIs bind to the Q_o site, preventing the movement of electrons between cytochrome b and cytochrome c_1 as well as stopping the synthesis of ATP (Bartlett et al., 2002). Future field study is needed to test a large number of fungicides regarding their activity to reduce ergot body weight and EAs production.

Correlating the Physical Characteristics of Ergot Sclerotia with the Total Ergot Alkaloids

The results presented in Table10 showed the correlation between the ergot-sclerotia parameters and the natural saprophytic *Fusarium* toxins with total EAs. The findings showed that increasing the total plot weight (clean and unclean samples) had a weak, negative non-significant correlation (-0.009, $P>0.05$) with total EAs production. In addition, the ergot body weight had a weak inverse correlation (-0.280) with total EAs, which means that the total amount of ergot body may not explain the total amount of EAs production.

The results also indicated that the ergot sclerotia's length had a weak, negative, and non-significant (-0.162) correlation with the total EAs, meaning that the increased in ergot sclerotia

length did not appear to be associated with the reduced total EAs. Also, the ergot sclerotia's width showed a weak, positive, and non-significant (0.091) correlation with the total EAs, which indicated that the greater width for the ergot sclerotia was not associated with the increased total EAs. Furthermore, there was a weak, positive, non-significant relationship between the weight of the ergot sclerotium and the total EAs (0.145).

Young (1981) found that there was no correlation between the sclerotia size and total percentage of alkaloids content and attributing the "bulk effect" as a reason for big sclerotia to contain more EAs. In addition, a study by Blaney et al. (2009) discovered that total alkaloids content did not change the length of Australian rye ergot sclerotia that was collected from the contaminated samples of wheat, grain, barley, ryegrass, and pasture. There were 13 samples that had a mean length of 6 mm, and the average total EAs was 2,585 mg/kg. Nine samples had a mean length of 7mm, and the average total EAs was 2,620 mg/kg; one sample had a mean length of 10 mm, and the average total EAs was 3,168 mg/kg.

Babič et al. (2020) found that the mean for the ergot-sclerotia weight was 134 mg in grain samples, and there was a weak, significant positive correlation between the ergot sclerotia's mass and the total EAs concentration, concluding that the increased ergot mass was not associated with an increase for the total EAs because of the size and weight differences of the individual sclerotium. Mainka et al. (2007) found that ergot sizes in the rye samples did not correlate with the total EAs, describing how most of the small, medium, and large sclerotia in the rye samples did not differ significantly in the amount of the total EAs.

For the ergot sclerotia in this study a weak, positive non-significant relationship between the weight of the ergot sclerotium and total EAs was found. However, Grusie et al. (2017) measured alkaloids in wheat containing different amounts of sclerotia, and found that the total

weight of ergot sclerotia had a positive and significant relationship (R^2=0.96, P<0.0001) with total EAs in 25 grain samples containing ergot sclerotia. The total EAs ranged from 10 to 22,521 ppb, and no significant relationship was found between the ergot sclerotia's weight and the total EAs when the total alkaloids' concertation was less than 350 ppb in 16 grain samples. It is important to note that the current study, measured the total EAs from ergot sclerotia that were separated from the CMS-HRS wheat, and the weight was determined as an average for the individual ergot sclerotium. While previous studies report the weight of ergot sclerotia in a grain sample and EAs content in grain samples containing ergot sclerotia.

Correlations Between the Compositional Characteristics of Ergot Sclerotia and Total Ergot Alkaloids

The moisture content of ergot sclerotia in relation to the production of total EAs is presented in Table10. The results indicated that moisture content had a weak and inverse, non-significant correlations (-0.021). with the total EAs. As indicated earlier, no previous study examined the relationship between ergot sclerotia's moisture content and the total EAs. However, the moisture content was discussed with other fungi. Grabowski et al. (2012) found that there was a weak, negative relationship between FHB infection and moisture content; the kernels of the infected wheat showed less moisture content, ranging from 6 to 8%, compared to the control. The authors explained that the decreased in moisture content might be due to the utilization of the fungi to the moisture during growth. Conversely, for another fungus Surovy et al. (2020) indicated that moisture contents were rose as the severity of the *Magnaporthe oryzae Triticum* (*MoT*) infection increased for the wheat samples, and the moisture content ranged from 18.36% to 18.73%.

Table 10. Simple linear correlations among ergot sclerotia's parameters of CMS-HRS wheat with total EAs (n= 20).

Parameters [a]	Total EAs
TPW	-0.009
EBW	-0.280
LTH	-0.162
WTH	0.091
WGT	0.145
Moisture content	-0.021
Ash content	0.110
Protein content	-0.161
EAs	1.000
DON	-0.263
NIV	-0.084
FUX	0.141
3-ADON	0.046
15-ADON	0.193
DAS	-0.502*
ENNS	0.151
HT2	0.459*
T-2	-0.044
ZEN	0.096

[a]TPW= Total plot weight, EBW= Ergot body weight, LTH= Length of ergot sclerotium, WTH=Width of ergot sclerotium, WGT= Weight of ergot sclerotium, EAs= Total ergot alkaloids, DON= Deoxynivalenol, NIV= Nivalenol, FUX= Fusarenon-x, 3-ADON= 3-acetyl deoxynivalenol,15-ADON=15-acetyl deoxynivalenol, DAS= Diacetoxyscirpenol, ENNS= Neosolaniol, and ZEN= Zearalenone.
*correspond to $\alpha = 0.05$.

In addition, ash content had a weak, positive, and non-significant (0.110) correlation with the total EAs. On the other hand, the protein content showed a weak, negative, and non-significant (-0.161) correlation with the total EAs. This study showed the ergot sclerotia's protein, ash, or moisture contents may not explain the amount of total EAs.

Correlations Between the Saprophytic *Fusarium* Toxins of Ergot Sclerotia and Total Ergot Alkaloids

Deoxynivalenol content had a weak, negative, and non-significant correlation (-0.263, $P>0.05$) with the total EAs (Table 10). However, among trichothecene group A, only DAS and HT-2 had a significant correlation with total EAs. Diacetoxyscirpenol showed a moderate, negative, and significant correlation (-0.502, $P<0.05$) with the total EAs. In addition, HT-2 toxin presented a moderate, positive and significant correlation with the total EAs (0.459, $P<0.05$). Moreover, the data presented non-significant correlations between the other trichothecenes of group B with the total EAs or ZEN with total EAs.

CONCLUSION AND FUTURE IMPLICATIONS

This study evaluated the effect of two toxins, DON and EAs contents, for the first time on the HRS wheat-quality parameters. From the perspective of food safety, these toxins will continuously threaten human and animal health. In this study, DON and EAs contents were detected in HRS-wheat samples in some regions, with other regions containing low or high levels of one or both or none of these toxins. Also, data showed that the influence of year or year and location interaction might not be significant for DON or total EAs contents. However, both toxins showed that location had a significant effect on their variations within one individual year. The total mean value for DON content during the 2019 growing season (1.5 ppm) was higher than the 2020 (0.6 ppm) growing season. The 1.5 ppm of DON level in 2019 was within the advisory level set by the FDA if wheat will undergo further processing. However, if this is the finished product, then 1.5 ppm of DON level is considered higher than the advisory level (NGFA, 2011).

Moreover, the total mean value for the total EAs in the 2019 growing season (81.5 ppm) was within the permissible limit for whole wheat flour established by the EU. Nevertheless, the total mean value in the 2020 growing season (180.55 ppb) exceeded the recommended level established by the EU (150 ppb) for milling products with an ash content above 0.9% in whole grain flour (Official Journal for the European Union, 2021). Consequently, this might cause an issue when exporting wheat to EU countries.

It is possible the presence of both toxins could affect the human body in the short or long term. Therefore, future research should be focused on how these toxins together could pose food safety risks, especially for consumers with high consumption of cereals. Risk assessment for individual and collective *fusarium* toxins in chestnut were conducted to evaluate the health risk

when adult and youth were exposed to such toxins (Liang et al., 2021). In addition, Yang et al. (2021) performed a risk assessment study for DON in wheat products collected from different areas in China. Children and adults exposed to wheat products tainted with DON were evaluated for a possible health risk (Yang et al., 2021).

Additional control strategies should be applied to protect humans and animals from possible toxicity, such as agronomic management including the careful use of fungicide to control mycotoxins. Beyond the use of fungicides, other control measures may include degrading EAs into less toxic compounds using UV light, heat, heat and humidity, and solvents (Schummer et al., 2020).

In terms of wheat quality, the results for the grading factors of for the HRS-wheat survey samples revealed that there might be a significant interaction effect of year and growing location on test weight. In addition, location had a significant effect on the variations of DHV kernels and test weight within one year or across the years. However, no such effects were found in the variations of shrunken and broken kernels, foreign matter, damaged kernels, and total defects.

The results of the non-grading factors for the HRS-wheat survey samples indicated that location had significant effects on the variations of moisture content, protein contented, and falling number within the year or across the years. Location had significant effects on the variation of thousand-kernel weight within one year. In the last part of this study, the observation of the correlations between DON and total EAs concentrations revealed that among the grading factors for the HRS-wheat survey samples, DON and EAs shared positive and significant correlations with total defects. In addition, DON was significantly correlated with test weight and damaged kernels. On the other hand, total EAs was significantly correlated with DHV kernels and foreign matter. Among the non-grading factors, DON and EAs had significant

correlations with moisture contents. In addition to the moisture contents, the level of EAs was significantly correlated with dockage, protein content, and 1,000-kernels weight, whereas DON content was significantly correlated with falling number.

For future research, it is recommended to implement a multi-year study to compare EAs and DON with wheat quality parameters. The correlation found between DON and total EAs with the grading and non-grading factors for the HRS-wheat survey samples could be investigated on flour data or dough properties to test the quality of the final products.

Another purpose of this study was to compare the toxin compositions of ergot sclerotia separated from CMS-HRS wheat after a single application of four different fungicides, at Feekes 10.5 prior to anthesis. The current study demonstrated that ergot sclerotia for all treatments had low moisture contents, had very high protein contents, and had high ash contents. Ergot body weight was significantly reduced when utilizing the four fungicides compared to the non-treated control. However, no common fungicide controlled the productions of DON (by the saprophytic *Fusarium*) and total EAs in ergot sclerotia. This study found that fungicides contained triazoles (Miravis Ace, Sphaerex and Quilt) increased the level of EAs compared to the non-treated control, but not significantly. Quilt was repeatedly tested in previous studies and showed effective results against *C.purpurea*. However, in our study, Quilt had a significantly lower ergot body weight compared to the control, but Quilt increased the level of EAs.

A single Priaxor application had a more profound effect on EAs content compared to the other fungicides which contained triazoles. Therefore, Priaxor fungicide could be a potential fungicide to suppress ergot sclerotia and EAs production. Miravis Ace treated samples performed better to suppress the DON contents of the saprophytic *Fusarium* in ergot sclerotia,

which was consistent with a previous study by (Singh et al., 2021) regarding Miravis Ace's ability to control the DON content.

This study also determined that ergot body weight had a weak, inverse correlation with the total EAs, which suggested that there was no relationship between ergot body weight and the total EAs production. In addition, no significant correlations were found between the ergot sclerotia's length, width, or individual weight with the total EAs.

Future research is needed to understand the effect of other fungicides not mentioned in this study to influence ergot body weight and the level of EAs. Further work should focus on the fungicides' influence on the EAs' toxicity level along with the time and the number of applications that are necessary to control ergot sclerotia in the fields.

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APPENDIX

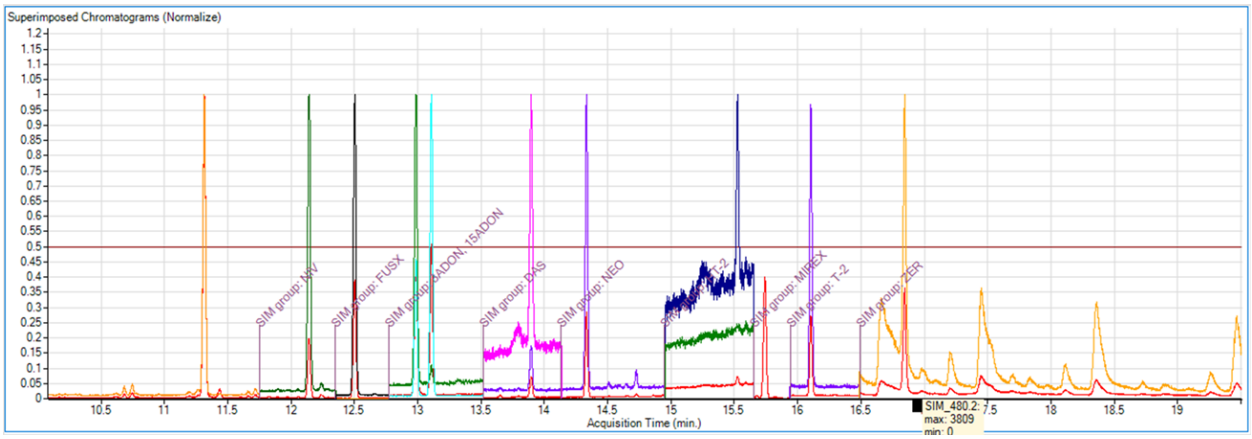

Figure A1. Mycotoxin standard mixture chromatogram for HRS wheat.

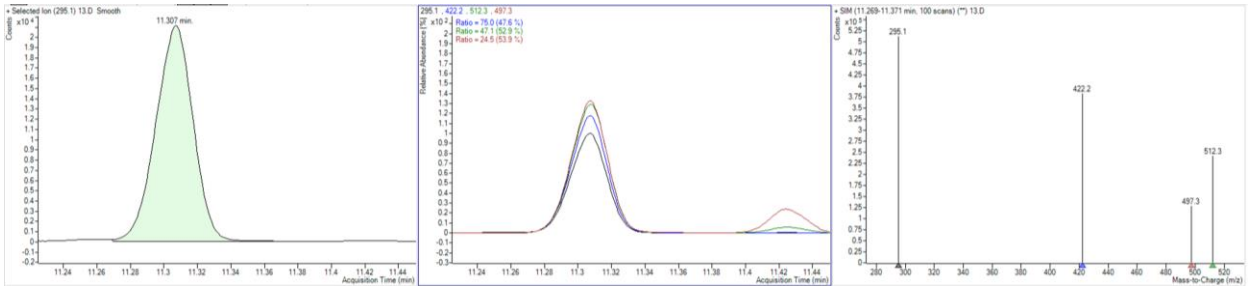

Figure A2. Deoxynivalenol chromatogram and spectra for HRS wheat.
From L to R: Compound peak, qualifier ion peak ratios, mass spectra.HRS wheat samples with at least 2.0 mg/kg of DON in GC/MS

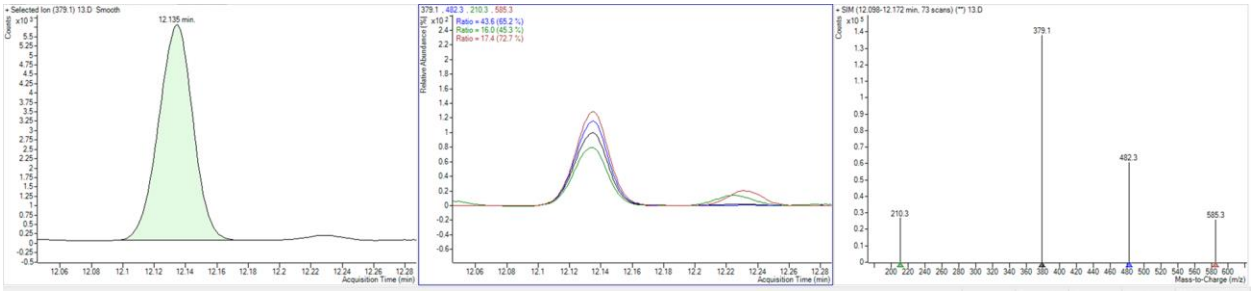

Figure A3. Nivalenol chromatogram and spectra for HRS wheat.

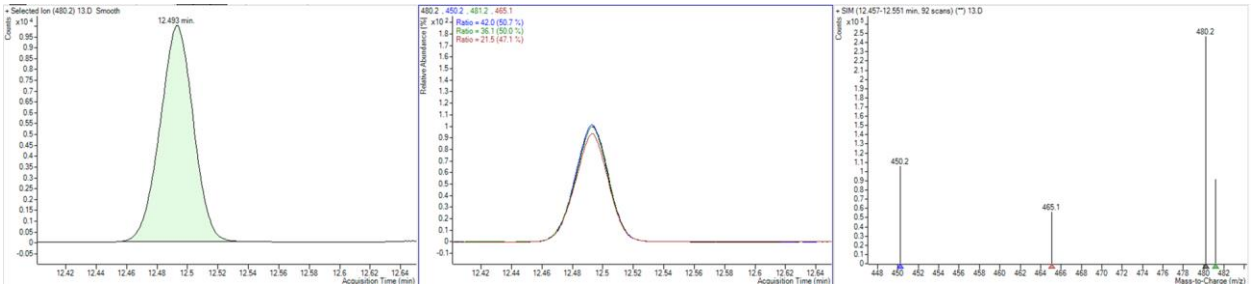

Figure A4. Fusarenone X chromatogram and spectra for HRS wheat.

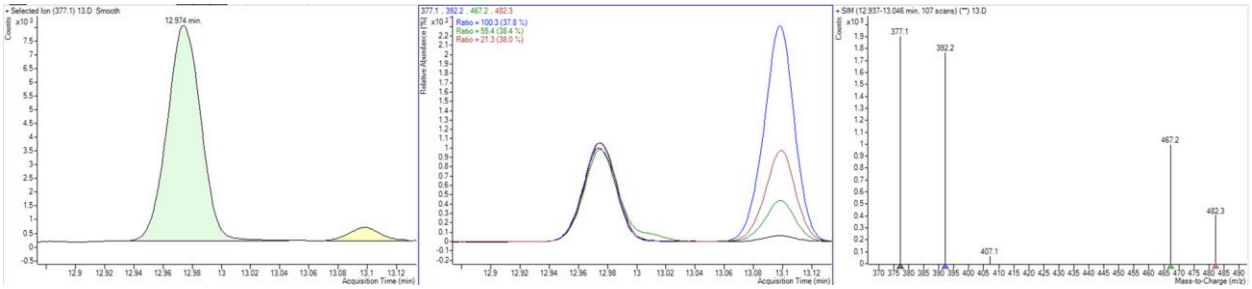

Figure A5. 3-acetyl-deoxynivalenol chromatogram and Spectra for HRS wheat.

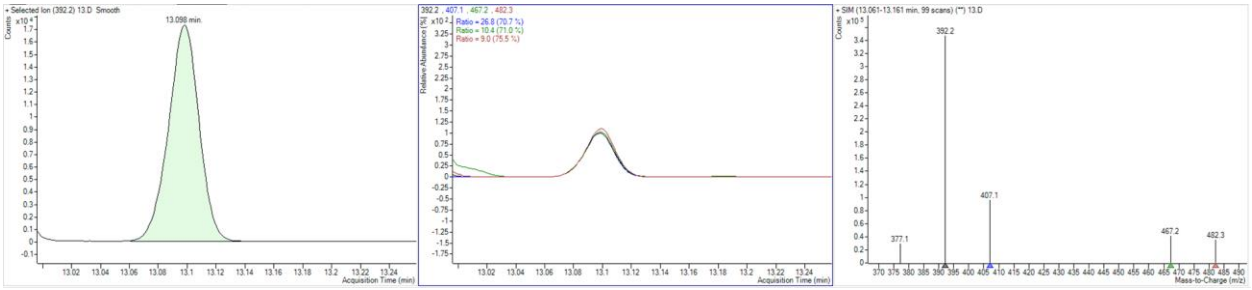

Figure A6. 15-acetyl-deoxynivalenol chromatogram and spectra for HRS wheat.

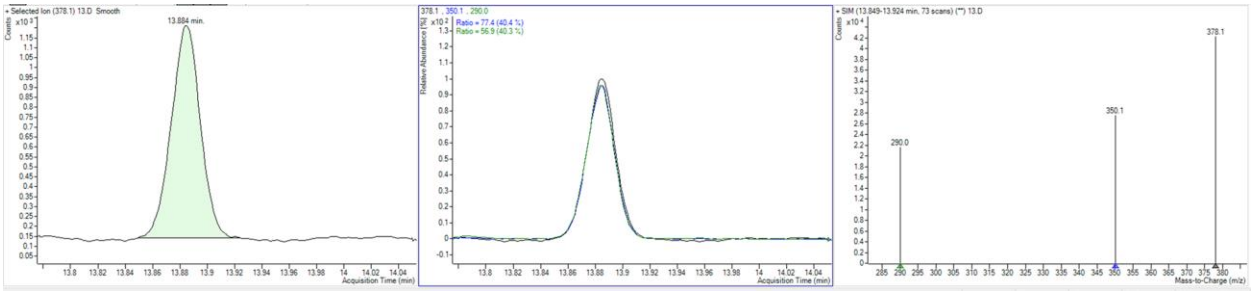

Figure A7. Diacetoxyscirpenol chromatogram and spectra for HRS wheat.

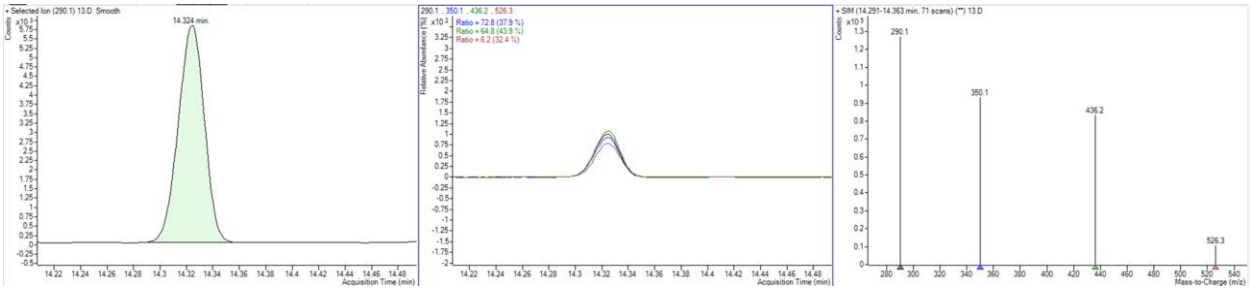

Figure A8. Neosolaniol chromatogram and spectra for HRS wheat.

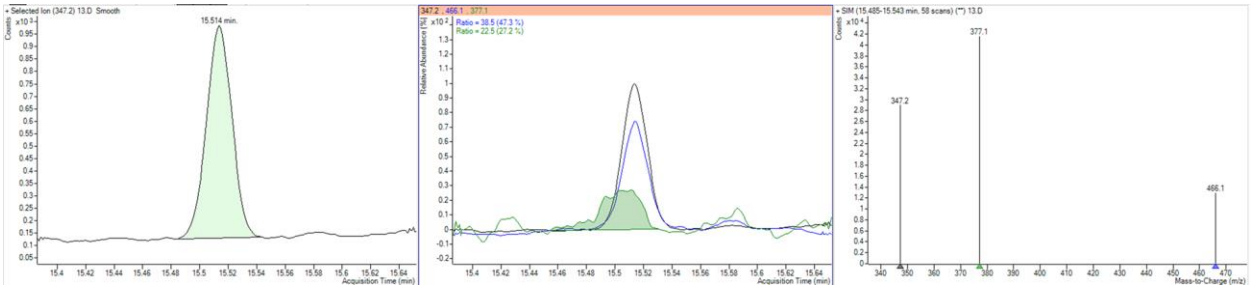

Figure A9. HT-2 Toxin chromatogram and spectra for HRS wheat.

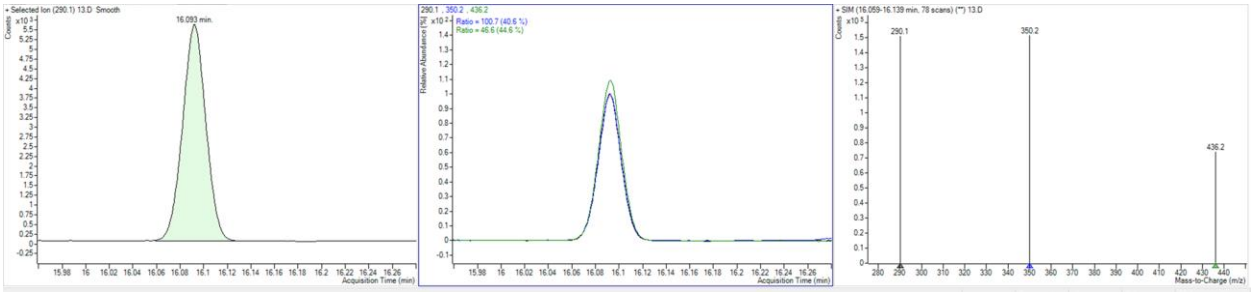

Figure A10. T-2 Toxin chromatogram and spectra for HRS wheat.

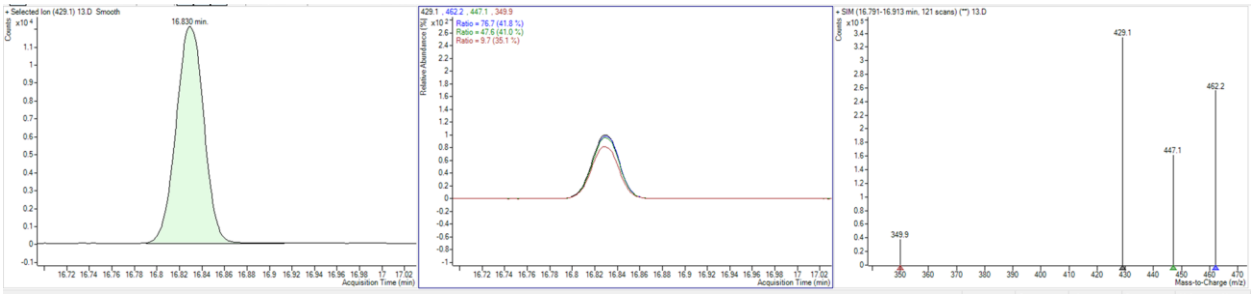

Figure A11. Zearalenone chromatogram and spectra for HRS wheat.

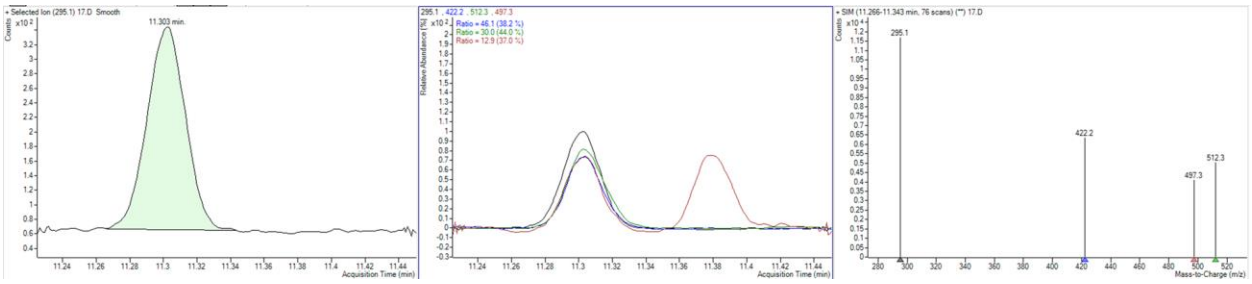

Figure A12. Deoxynivalenol chromatogram and Spectra for ergot sclerotia.

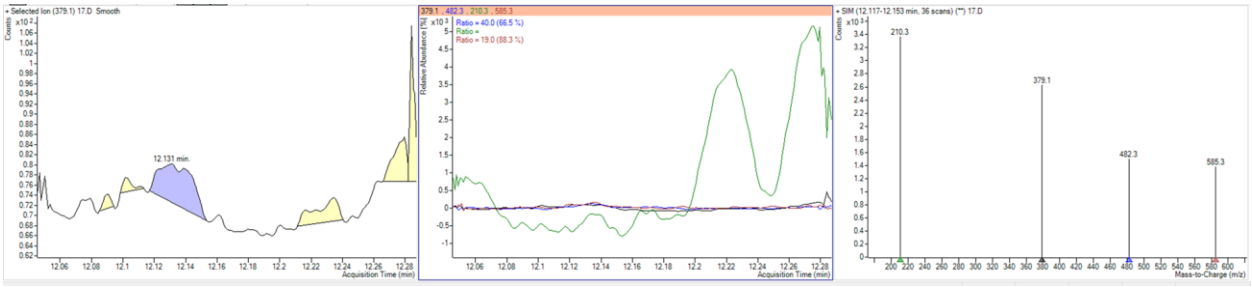

Figure A13. Nivalenol chromatogram and spectra for ergot sclerotia.

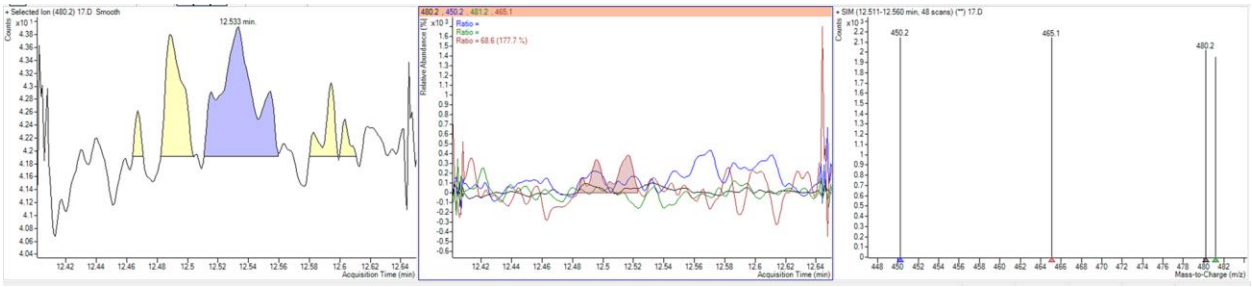

Figure A14. Fusarenone-X chromatogram and spectra for ergot sclerotia.

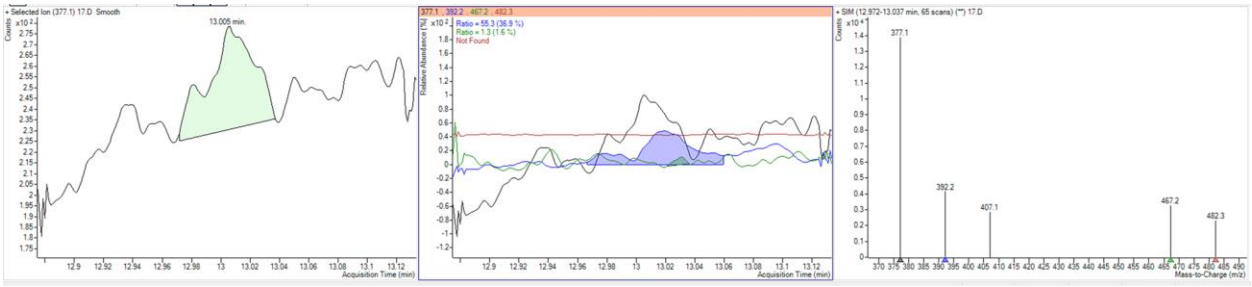

Figure A15. 3-acetyl-deoxynivalenol chromatogram and spectra for ergot sclerotia.

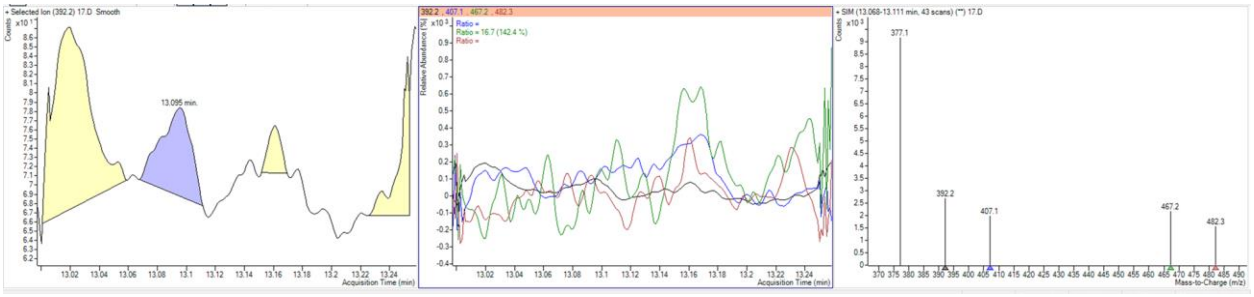

Figure A16. 15-acetyl-deoxynivalenol chromatogram and spectra for ergot sclerotia.

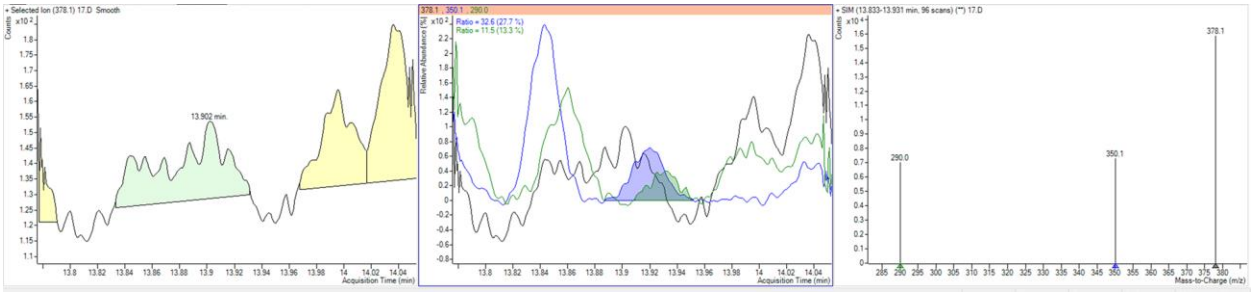

Figure A17. Diacetoxyscirpenol chromatogram and Spectra for ergot sclerotia.

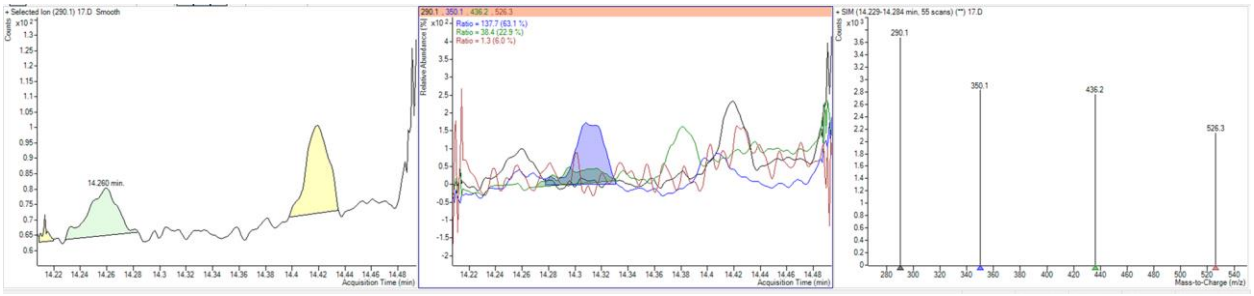

Figure A18. Neosolaniol chromatogram and spectra for ergot sclerotia.

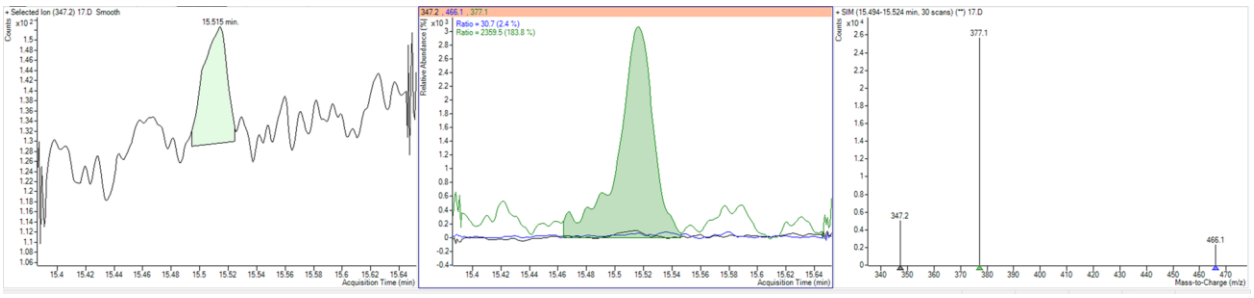

Figure A19. HT-2 Toxin chromatogram and spectra for ergot sclerotia.

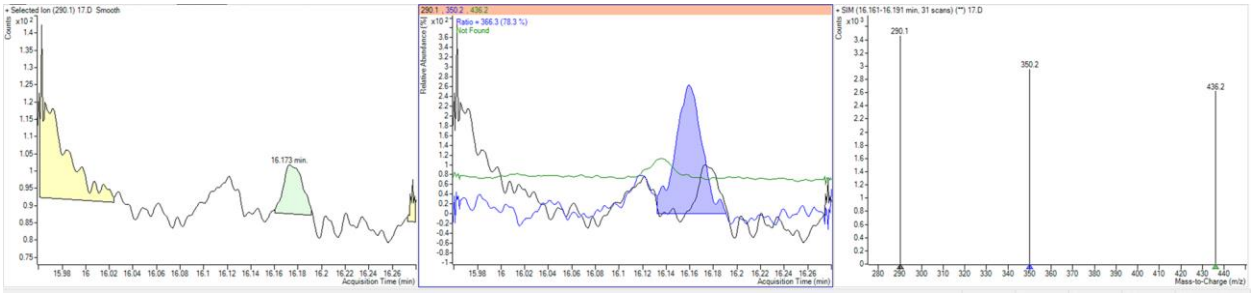

Figure A20. T-2 Toxin chromatogram and spectra for ergot sclerotia.

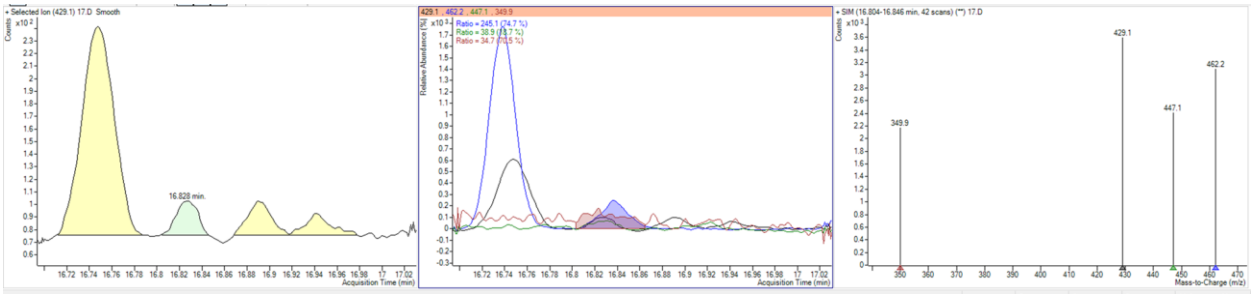

Figure A21. Zearalenone chromatogram and spectra for ergot sclerotia.