FATE OF DEOXYNIVALENOL DURING WET MILLING

A Thesis
Submitted to the Graduate Faculty
of the
North Dakota State University
of Agriculture and Applied Science

By

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In Partial Fulfillment of the Requirements
for the Degree of
MASTER OF SCIENCE

Major Program:
Cereal Science

September 2018

Fargo, North Dakota

North Dakota State University
Graduate School

Title

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

MASTER OF SCIENCE

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ABSTRACT

The fungal disease Fusarium head blight affects cereal grains and can produce mycotoxins, like the water-soluble deoxynivalenol (DON). Wheat wet milling process begins with ground endosperm obtained by dry milling and ends with the separation of starch from gluten. Research was conducted on hard red spring wheat and durum wheat samples naturally contaminated with DON. The fate of DON in wheat dry milled fractionations (farina/semolina, shorts, and bran) during wet milling was investigated. Three wet milling processes were evaluated. DON levels were assessed by GC-ECD. Results showed that DON was present in all dry milled fractions. DON concentration in farina and semolina exceeded the safety threshold for human consumption. After wet milling farina and semolina, nearly all the DON was found in the water-soluble fraction, regardless the wet milling process. A negligible level of DON was found in the gluten extracted from HRSW with Martin wet milling process.

ACKNOWLEDGEMENTS

Firstly, I would like to express my sincere gratitude to my advisor Dr. Senay Simsek. Thank you for accepting me as your student, for encouraging me to reach out and attend international meetings exposing our work. Your guidance and motivation have helped me to successfully conduct this research and develop my professional life.

My deep appreciation extends to Dr. Manthey. Thank you for all the guidance, advice, and support provided during this experience. Thank you for providing me a working space in your lab. Thank you for always having the door open whenever I ran into a trouble spot or had a question about my research and writing. Without your support the conduction of this research would not have been possible.

I have endless gratitude for my committee members Dr. Linda Dykes and Dr. Saleem Shaik, for their manuscript revisions, insightful comments and assistance provided during my Master studies.

I would like to show my gratitude to Dr. Jae B. Ohm for his assistance in the statistical analysis and his patience with my constant questions.

My gratitude also extends to Kristin Whitney and Yu Liu (Gabby) for their immense knowledge, support, and for sharing their excellent professional skills while conducting this research.

My thanks extend to my NDSU lab-mates, office-mates, and friends for their constant support and for making grad school one of the most extraordinary stages in my life.

I would also like to thank Antonio Molina for being the best partner and friend anyone could ask for. Thank you for sharing this adventure from the very beginning, for believing in me, and for offering me endless support and help throughout all the process.

Finally, I will never have enough words to express my immense gratitude to my family. My parents, Rosy López and Francisco Magallanes, have been my support through each stage in my life, and have given me the strength and necessary skills to fulfill my dreams. My brother, Paco Magallanes, has always been my confident, ally and friend, so thank you for always drawing a smile on my face.

DEDICATION

I would like to dedicate this thesis to my beloved parents,

Rosy López and Francisco Magallanes,

who I owe the person I am today.

I love you.

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LIST OF ABBREVIATIONS

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

DON ..... Deoxynivalenol

DW ..... Durum Wheat

FDA ..... Food and Drug Administration

FHB ..... Fusarium Head Blight

GC-ECD ..... Gas Chromatography with Electron Capture Detection

HRSW ..... Hard Red Spring Wheat

WWF ..... Whole Wheat Flour

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1. GENERAL INTRODUCTION

Fungi growing on foodstuffs are responsible for producing mycotoxins. The word ‘mycotoxin’ is derived from the Greek word ‘mykes’ meaning fungus and the Latin word ‘toxicum’ meaning poison (Turner, Subrahmanyam, & Piletsky, 2009). The most relevant mycotoxins, due to their feed-manufacturing importance, in cereal grains and the fungi genus that produce them are aflatoxins (*Asperillus*), trichothecenes (particularly deoxynivalenol [DON], *Fusarium*), fumonisins (*Fusarium*), zearalenone (*Fusarium*) and ochratoxins (*Aspergillus* and *Penicillium*) (Binder, Tan, Chin, Handl, & Richard, 2007; Krska, Welzig, & Boudra, 2007). Mycotoxins aflatoxins, ochratoxins, zearalenone, and DON are commonly associated with wheat (Alshannaq, & Yu, 2017).

In 1982, wheat growers from Idaho, Illinois, Iowa, Kansas, Missouri, Nebraska, and Oklahoma were impacted by a serious disease known as Fusarium head blight (FHB), which caused an estimated 4% reduction in wheat production nationwide (Boosalis, Doupnik Jr., Wysong, & Watkins, 1983). Additionally, Sutton (1982) reported that in Canada, FHB outbreaks in wheat occurred sporadically in wheat-growing areas like Ontario, Quebec, Manitoba, and Alberta, where in the period from 1927 to 1980 severe FHB were registered during 1940, 1942, 1945, 1957, 1967, and 1980. Furthermore, eastern North Dakota and western Minnesota spring and durum wheat crops were impacted by FHB in 1986 causing severe test weight reductions (McMullen, & Stack, 1987). The Wheat Disease Survey conducted in 1987 revealed that 19% fields in North Dakota were impacted by FHB (McMullen, Stover, Hosford, & Nelson, 1988).

Since 1993, North American regions subjected to summer rains have faced the re-emergence of this serious disease, considered as the worst plant disease to hit USA since 1950, when stem rust epidemics arose (Windels, 2000). Several factors have favored the disease spread, such as increased inoculum due to reduced tillage systems, monoculture of corn (*Zea mays* L.) or wheat (*Triticum aestivum* L.), as well as environmental changes, especially rainfall between anthesis and flowering (Dill-Macky, & Jones, 2000; Okubara et al., 2002).

The phytopathogenic agents *Fusarium graminearum* and *Fusarium culmorum* are responsible for FHB in wheat, causing negative effects on crop size and grain quality, as well as the accumulation of DON (Richard, 2007; Visconti, Haidukowski, Pascale, & Silvestri, 2004). The infection of grains, including

wheat, corn, oat (*Avena sativa* L.), and barley (*Hordeum vulgare* L.) occur because *Fusarium* survives on residue of the previous crop. Fungal spores germinate and release conidia that are carried by the wind to small grain anthers during anthesis, or to developing grain and forms hyphae that penetrates into the floret or grain, resulting in the disease condition known as FHB (Richard, 2007).

Fusarium uses DON to disturb the plant defense system at the infection critical stages to assure the colonization and symptom development (Audenaert, Vanheule, Höfte, & Haesaert, 2013). DON has been shown to inhibit protein synthesis *in vitro* (Yuan et al., 1999). DON synthesis during late growing season is induced by certain environmental conditions (Richard, 2007), such as temperature (21-23°C) and high moisture levels (Del Ponte, Fernandes, & Bergstrom, 2007).

DON has been shown to have a negative impact in animals. DON can cause immunosuppression, cell necrosis, and smooth muscle paralysis in animals (Okubara et al., 2002; Richard, 2007). Furthermore, DON is known as vomitoxin due to its strong emetic effect after food or feed consumption, which is explained by the transport of DON to the brain, where it induces the activation of dopaminergic receptors (Sobrova et al., 2010). In humans, DON mycotoxicosis may be manifested by diarrhea, nausea, vomiting, dizziness, and fever (Pestka, 2007; Sobrova et al., 2010). Therefore, regulatory guidelines of maximum levels of DON in feed and food have been released. For instance, the FDA established DON limits as follow: one ppm in finished wheat products for human consumption, whereas 10 ppm in grain and grain byproducts for ruminating beef and feedlot cattle, and 5 ppm in grain and grain byproducts for swine and all other animals (Wegulo, 2012).

Grain and food processing represents opportunities to reduce the concentration of mycotoxins in cereal grains and ensure consumer safety. Grain processing includes cleaning to remove diseased kernels and dry milling to separate the bran and germ from the endosperm. Since most of the harvested wheat is dry milled, some studies about the impact of milling and the fate of DON have been published (Cheli et al., 2010; Dexter, Marchylo, Clear, & Clarke, 1997; Ríos, Pinson-Gadais, Abecassis, Zakhia-Rozis, & Lullien-Pellerin, 2009; Tibola, Cunha Fernandes, Guarienti, & Nicolau, 2015). The studies concluded that DON is not eliminated during the milling process. Milling separates the bran and germ from the endosperm. DON in the refined flour is associated with the endosperm. In general, DON

concentrations are lower in flour or semolina than in bran or germ (Vidal, Sanchis, Ramos, & Marín, 2016).

DON is stable at temperatures up to 120°C and moderately stable at 180°C, and it is soluble in water and polar solvents, such as aqueous methanol, acetonitrile and ethyl acetate (Döll, & Dänicke, 2011). These results indicate that DON would be stable during most processes associated with baking and pasta/noodle processing. For instance, in the bread baking process during fermentation, DON concentration has been reported as not affected, increased, or conjugated forming masked mycotoxins (Khaneghah, Martins, von Hertwig, & Bertoldo, 2018). Investigations have shown reduction of DON during noodle or spaghetti cooking in boiling water (Visconti et al, 2004; Visconti, & Pascale, 2010), taking advantage of DON water solubility. These reports suggest the possibility of removing DON utilizing processes that require excess water, such as a wet milling process.

In contrast with dry milling process, wet milling process is applied to achieve the separation of the main chemical components (protein, starch, lipid, and fiber) of different plants, like corn or wheat, by the application of physical, chemical, biochemical, and mechanical operations (Wronkowska, 2016). Commercially, wheat is wet milled to produce gluten protein and starch for industrial purposes. Wet milling processes starting with wheat flour rather than wheat kernels are employed commercially; these processes include: Martin (dough-washing), Alfa-Laval/Raisio, Hydrocyclone and High-Pressure Desintegration processes (Sayaslan, Seib, & Chung, 2012). Wet separation and purification of gluten protein and starch granules from wheat flour are based on differences in their aggregation, particle sizes and sedimentation rates (Sayaslan, 2004; Van Der Borght, Goesaert, Veraverbeke, & Delcour, 2005). The isolated gluten protein fraction from wheat can be called ‘vital gluten’ if it is still functional, where it can form a viscoelastic gluten network (Van Der Borght et al., 2005).

The question arises as to whether or not DON can be removed or reduced from starch and protein during wet milling; thus making the fractionated starch and protein eligible to be used as food ingredients. In this context, the main objectives of this study were as follows,

1. To determine if DON can be removed from starch and protein fractions of Hard Red Spring (HRS) and durum wheat samples by using the traditional Martin wet milling process.

2. To compare the ability of different wet milling processes (Martin process, moderately sheared process, and highly sheared process) to remove DON from farina/semolina.

1.1. References

Alshannaq, A., & Yu, J.-H. (2017). Occurrence, toxicity, and analysis of major mycotoxins in food. *International Journal of Environmental Research and Public Health*, *14*, 632.

Audenaert, K., Vanheule, A., Höfte, M., & Haesaert, G. (2013). Deoxynivalenol: A major player in the multifaceted response of Fusarium to its environment. *Toxins*, *6*, 1-19.

Binder, E. M., Tan, L. M., Chin, L. J., Handl, J., & Richard, J. (2007). Worldwide occurrence of mycotoxins in commodities, feeds and feed ingredients. *Animal Feed Science and Technology*, *137*, 265-282.

Boosalis, M. G., Doupnik Jr., B. L., Wysong, D. S., & Watkins, J. E. (1983). The wheat scab epidemic of 1982. *Farm, Ranch and Home Quarterly*, *29*, 7-9.

Cheli, F., Campagnoli, A., Ventura, V., Brera, C., Berdini, C., Palmaccio, E., & Dell'Orto, V. (2010). Effects of industrial processing on the distributions of deoxynivalenol, cadmium and lead in durum wheat milling fractions. *Journal of Food Science and Technology*, *43*, 1050-1057.

Del Ponte, E. M., Fernandes, J. M., & Bergstrom, G. C. (2007). Influence of growth stage on Fusarium Head Blight and deoxynivalenol production in wheat. *Journal of Phytopathology*, *155*, 577-581.

Dexter, J. E., Marchylo, B. A., Clear, R. M., & Clarke, J. M. (1997). Effect of fusarium head blight on semolina milling and pasta making quality of durum wheat. *Cereal Chemistry*, *74*, 519-525.

Dill-Macky, R., & Jones, R. K. (2000). The effect of previous crop residues and tillage on Fusarium head blight of wheat. *Journal of Plant Diseases and Protection*, *84*, 71-76.

Döll, S., & Dänicke, S. (2011). The Fusarium toxins deoxynivalenol (DON) and zearalenone (ZON) in animal feeding. *Preventive Veterinary Medicine*, *102*, 132-145.

Khaneghah, A. M., Martins, L. M., von Hertwig, A. M., & Bertoldo, R. (2018). Deoxynivalenol and its masked forms: Characteristics, incidence, control and fate during wheat and wheat based products processing – A review. *Trends in Food Science & Technology*, *71*, 13-24.

Krska, R., Welzig, E., & Boudra, H. (2007). Analysis of Fusarium toxins in feed. *Animal Feed Science and Technology*, *137*, 241-264.

McMullen, M. P., & Stack, R. W. (1987). Improving wheat and durum seedling stands and vigor by fungicide seed treatments. *Farm Research*, *44*, 13-15.

McMullen, M. P., Stover, R. W., Hosford Jr., R. M., & Nelson, D. R. (1988). 1987 Wheat disease survey results indicate disease potential for ’88. *Farm Research*, *45*, 3-6.

Okubara, P. A., Blechi, A. E., McCormick, S. P., Alexander, N. J., Dill-Macky, R., & Hohn, T. M. (2002). Engineering deoxynivalenol metabolism in wheat through the expression of a fungal trichothecenes acetyltransferase gene. *Theoretical and Applied Genetics*, *106*, 74-83.

Pestka, J. J. (2007). Deoxynivalenol: Toxicity, mechanisms and animal health risks. *Animal Feed Science and Technology*, *137*, 283-298.

Richard, J. L. (2007). Some major mycotoxins and their mycotoxicoses- An overview. *International Journal of Food Microbiology*, *119*, 3-10.

Ríos, G., Pinson-Gadais, L., Abecassis, J., Zakhia-Rozis, N., & Lullien-Pellerin, V. (2009). Assessment of dehulling efficiency to reduce deoxynivalenol and fusarium level in durum wheat grains. *Journal of Cereal Science*, *49*, 378-392.

Sayaslan, A. (2004). Wet-milling of wheat flour: Industrial processes and small-scale test methods. *Journal of Food Science and Technology*, *37*, 499-515.

Sayaslan, A., Seib, P. A., & Chung, O. K. (2012). A bench-scale high-shear wet-milling test for wheat flour. *Journal of Food Engineering*, *11*, 305-317.

Sobrova, P., Adam, V., Vasatkova, A., Beklova, M., Zeman, L., & Kizek, R. (2010). Deoxynivalenol and its toxicity. *Interdisciplinary Toxicology*, *3*, 94-99.

Sutton, J. C. (1982). Epidemiology of wheat head blight and maize ear rot caused by Fusarium graminearum. *Canadian Journal of Plant Pathology*, *4*, 195-209.

Tibola, C. S., Cunha Fernandes, J. M., Guarienti, E. M., & Nicolau, M. (2015). Distribution of Fusarium mycotoxins in wheat milling process. *Food Control*, *53*, 91-95.

Turner, N. W., Subrahmanyam, S., & Piletsky, S. A. (2009). Analytical methods for determination of mycotoxins: A review. *Analytica Chimica Acta*, *632*, 168-180.

Van Der Borght, A., Goesaert, H., Veraverbeke, W. S., & Delcour, J. A. (2005). Fractionation of wheat and wheat flour into starch and gluten: overview of the main processes and the factors involved. *Journal of Cereal Science*, *41*, 221-237.

Vidal, A., Sanchis, V., Ramos, A. J., & Marín, S. (2016). The fate of deoxynivalenol through wheat processing to food products. *Food Science*, *11*, 34-39.

Visconti, A., Haidukowski, E. M., Pascale, M., & Silvestri, M. (2004). Reduction of deoxynivalenol during durum wheat processing and spaghetti cooking. *Toxicology Letters*, *153*, 181-189.

Visconti, A., & Pascale, M. (2010). An overview on Fusarium mycotoxins in the durum wheat pasta production chain. *Cereal Chemistry*, *87*, 21-27.

Windels, C. E. (2000). Economic and social impacts of Fusarium head blight: Changing farms and rural communities in the northern Great Plains. *Phytopathology*, *90*, 17-21.

Wegulo, S. N. (2012). Factors influencing Deoxynivalenol accumulation in small grain cereals. *Toxins*, *4*, 1157-1180.

Wronkowska, M. (2016). Wet-milling of cereals. *Journal of Food Processing and Preservation*, *40*, 572-580.

Yuan, Q., Pestka, J. J., Hespenheide, B. M., Kuhn, L. A., Linz, J. E., & Hart, L. P. (1999). Identification of mimotope peptides which bind to the mycotoxin deoxynivalenol-specific monoclonal antibody. *Applied and Environmental Microbiology*, *65*, 3279-3286.

2. LITERATURE REVIEW

Cereal grains represent one of the principal sources of calories for the majority of the world population. In developing countries, around 60% of the caloric intake comes from cereals, in contrast with developed countries where only 30% of the calories are derived from cereals (Wronkowska, 2016). Wheat is one of the three major cereal crops grown worldwide with over 750 million tonnes produced in 2016 (FAO, 2018). The term 'wheat' applies to a group of species which belong to the grass family and the genus *Triticum*. Archeological evidence shows that ancient wheat was domesticated in the Middle East around 10,000 years ago, its cultivation allowed the establishment of permanent settlements triggering civilization as we know it (Oleson, 1994). In several countries wheat is the major component of the diet since it is non-perishable, easy to transport, and allows the manufacture of a wide variety of products (Ranhotra, 1994).

2.1. Wheat Classification

The most important modern wheat species are hexaploid (AABBDD genome) bread wheat (*Triticum aestivum* L.) and tetraploid (AABB genome) durum wheat (*T. turgidum* L. var. *durum* H.) (Peña, 2002), which differ from their genomic make-up, and food end-use quality attributes, but denote roughly equivalent percentages of starch, protein, minerals, lipids, and amino acids (Matsuo, 1994). Wheat for trading purposes is classified into distinct categories based on grain hardness (soft to hard), color (red, white, or amber), and growing habitat (spring or winter) (Peña, 2002). Regarding hardness, common hexaploid wheat endosperm texture ranges from very soft to hard, whereas tetraploid durum wheat denotes the hardest kernels of all wheat cultivars (Pauly, Pareyt, Fierens, & Delcour, 2013). Hexaploid wheat is mainly used as flour for the production of baked products, such as bread, noodles, and cookies, whereas tetraploid durum wheat is used to obtain semolina so as to prepare pasta, and other Middle Eastern products like couscous (Peña, 2002).

2.1.1. Wheat Spike and Kernel Structure

The inflorescence of wheat is a spike (Figure 2.01) with a primary axis, or rachis, which is a sinuous notched structure composed of short internodes that bears two opposite rows of lateral secondary spikelets (Lersten, 1987; Percival, 1921). The rachis is tough and resists disarticulation even when trashed, on the other hand all the spikelets are sessile. Each spikelet is a condensed reproductive

shoot that has a short spikelet axis, or rachilla, where the glumes are arranged alternately on opposite sides (De Vries, 1971; Percival, 1921). The two bottom glumes are mostly sterile, and the next are fertile containing two to five florets per spikelet being every floret able to produce one seed (Percival, 1921). Each floret is enclosed by two bract-like structures, called lemma and palea. Between the lemma and palea are the sexual organs forming a perfect flower, which consists of three stamen and a single pistil, and two small lodicules. The stamen is formed by a filament and elongated anther containing pollen, whereas the pistil is an ovary with two short styles, each with feathery stigma branches (Figure 2.02) (De Vries, 1971; Lersten, 1987).

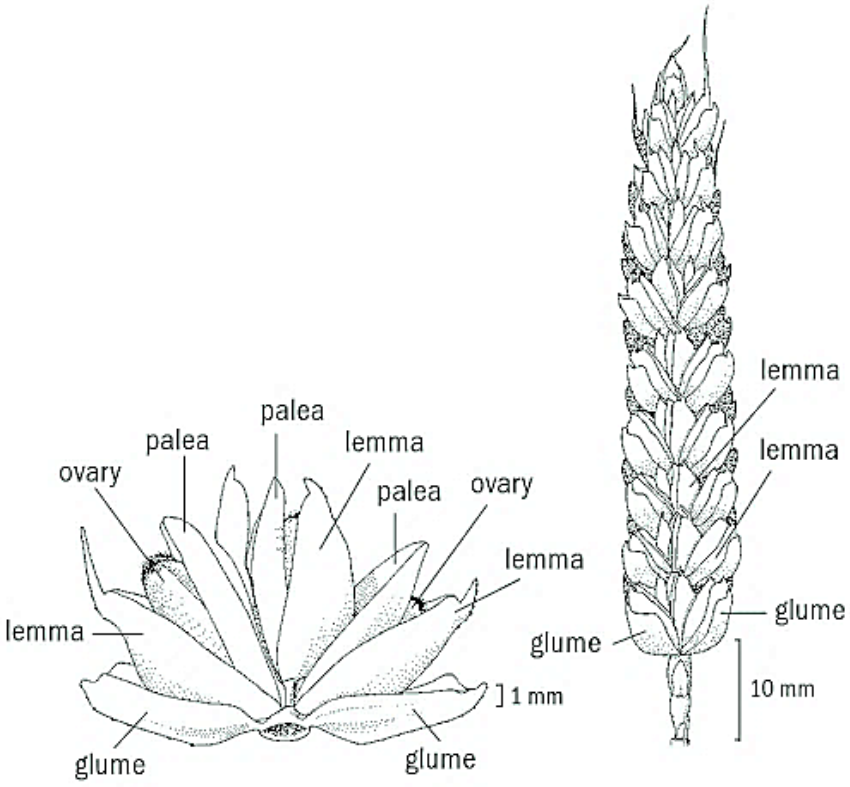

Figure 2.01. Wheat single spikelet parts and inflorescence spike.
(Adapted from Bell, 2008).

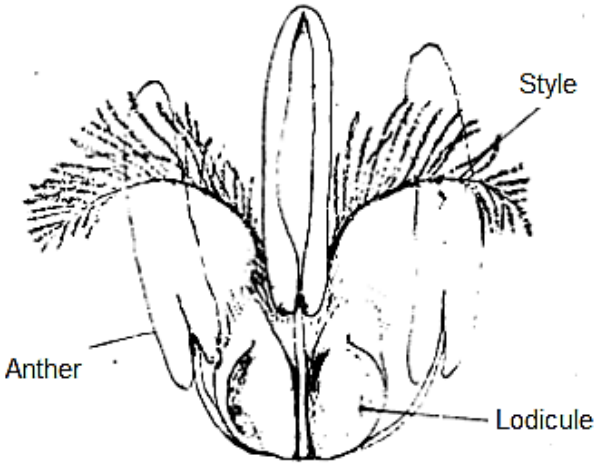

Figure 2.02. Wheat flower parts: anther, style and lodicule.
(Adapted from Percival, 1921).

After pollination and fertilization the number of cells in the endosperm is established. One to two weeks after pollination the kernel consistency turns into “soft dough” since the grain moisture content ranges from 40 to 45%, and the accumulation of starch and protein in the kernel occurs rapidly. Around three weeks after pollination, the kernel growth declines turning into a “hard dough” consistency as water content decreases to 32 to 35%. Finally, physiological maturity is met when the kernel reaches its maximum dry weight and becomes viable, and the moisture in the kernel declines rapidly below 15% (Pask, 2012; Simmons, Oelke, & Anderson, 1985). Ideally, grain is harvested when moisture content is 13.5% or less. The wheat kernel growth stages are shown in Figure 2.03.

Figure 2.03. Wheat kernels at grain filling stages: a) watery ripe; b) late-milk; c) soft dough; d) hard dough; and e) ripe for harvest.
(Adapted from Simmons et al., 1985).

The structure of the wheat kernel is shown in Figure 2.04. The dorsal side of the wheat grain is rounded, while the ventral side has a deep groove or crease along the longitudinal axis (Pomeranz, 1982). Seed is composed of three main parts: germ, bran, and endosperm. The wheat germ is located in the dorsal side, and it consists of rudimentary roots and shoots, and scutellum. At the opposite end of the kernel, there is a cluster of short fine hairs known as brush. Bran layer surrounds the seed and is composed of several layers (Posner, 2000). The outermost coat layer, pericarp or fruit coat, consists of outer pericarp, formed of outer epidermis, hypodermis, and thin-walled cells, and inner pericarp, formed of intermediate-size cells, cross layers, and tube cells. The inner coat layers are seed coat (testa) and nucellar epidermis. Between the nucellar epidermis and the starchy endosperm is the aleurone layer, which botanically is the outer layer of the endosperm, but as it tends to remain attached to the outer coats during wheat milling, so it is considered by millers as the innermost bran layer. Finally, the starchy endosperm consists of large starch granules surrounded by protein (Pomeranz, 1982; Posner, 2000).

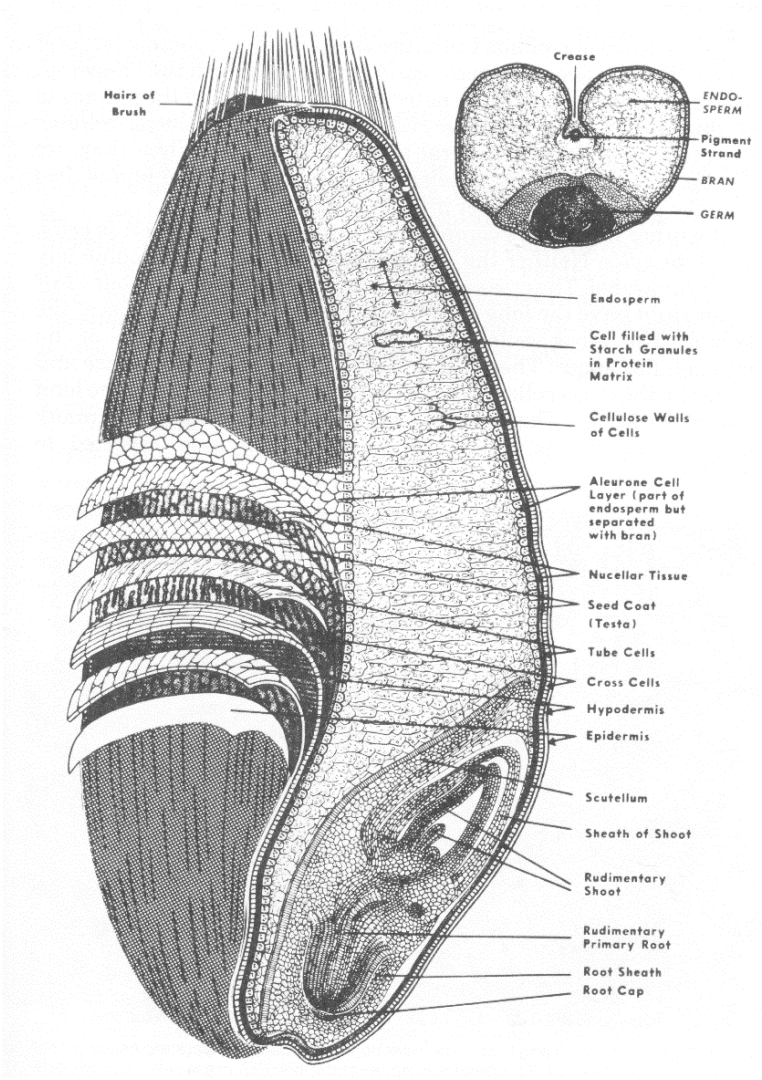

Figure 2.04. Wheat kernel in longitudinal and cross section.
(Adapted from Posner, 2000).

2.1.2. Wheat Kernel Chemistry

The wheat germ, which comprises around 2 to 3% of the grain, contains 10 to 15% lipids, 26 to 35% proteins, 17% sugars, and 4% minerals (Bradolini, & Hidalgo, 2012). Regarding the lipids, 2 to 6% are unsaponifiable matters that contains sterols, tocopherols, and pigments. The remaining saponifiable lipid contains 11 to 17% of palmitic acid, 0.6 to 3.6% of stearic acid, in addition to high levels of unsaturated fatty acids, such as, 14 to 25% oleic acid, 49 to 60% linoleic acid, and 4 to 10% linolenic acid; additionally, phospholipids constitute around 15% of germ lipids, whereas the amount of glycolipids is negligible (Ghafoor et al., 2017; Pomeranz, 1988). Germ protein types, as percentages of the total

protein, are around 30 to 34.5% albumin, 18.9 to 20% globulin, 13.9 to 15% gliadin, 0.3 to 0.4% glutenin, and 30% insoluble protein (Pomeranz, 1988).

Wheat bran as a by-product of wheat dry milling contains the bran layers, aleurone layer and underlying attached starchy endosperm which constitutes 13 to 17% of the grain. Wheat bran also contains 14 to 25% starch, 13 to 18% protein, 3 to 8% minerals, and 53 to 57% dietary fiber (Apprich et al., 2014; Sramková, Gregová, & Sturdík, 2009). Bran protein types, as percentages of the total protein, are approximately 18 to 23% albumins, 11 to 16% globulins, 9 to 18% prolamins, 19 to 26% glutelins, and 16 to 22% insoluble protein (Pomeranz, 1988). Additionally, compared to endosperm proteins, bran proteins contain higher amounts of lysine, arginine, alanine, and glycine, and less glutamine, proline, phenylalanine, and sulfur containing amino acids (Cornell, 2012; Sramková et al., 2009).

The two external layers, pericarp and seed coat, are made from dead empty cells, while the inner-layer, aleurone, is made from living cells (Sramková et al., 2009). In the aleurone layer enzyme activity from phytase, esterase, amylase, protease, dipeptidase, and dehydroascorbic acid reductase has been reported having a vital role in the germination process (Pomeranz, 1988). Additionally, wheat bran is a rich source of bioactive components like phenolic compounds, which consist of phenols with one aromatic ring represented by phenolic acids (Apprich et al., 2014). The dominant phenolic compound in wheat is ferulic acid, which 98% of its total proportion in the wheat kernel is located in the bran layer (Lempereur, Rouau, & Abecassis, 1997).

The mealy endosperm represents about 80 to 85% of the wheat kernel consisting of energy-yielding starch (64 to 74%), proteins (8 to 14%), lipids (1.5%), dietary fiber (1.5%), and ash (0.5%) (Cornell, 2012; Pomeranz, 1988). Starch is the major storage carbohydrate of cereals, it is present in the endosperm in the form of granules. Starch granules consist of two water-insoluble homoglucans, amylose and amylopectin formed in the amyloplast in the endosperm. Amylose consists of α-(1,4)-linked D-glucopyranosyl units and it is essentially linear. On the other hand, amylopectin is a highly branched polysaccharide consisting of α-(1,4)-linked D– glucopyranosyl chains, which are interconnected via α-(1,6)-glycosidic linkages, also called branch points (Koehler, & Weiser, 2013). Amylopectin is responsible for the granular nature of starch. Wheat starch granules are of two sizes: large, lenticular A-type granules

(15 to 40 μm), and small, spherical B-type granules (1 to 10 μm) (Van Der Borght et al., 2005). Normal wheat starch typically contains 20 to 30% amylose and 70 to 80% amylopectin (Sramková et al., 2009).

Regarding endosperm proteins, average content of wheat grain can vary from less than 6% to more than 20%; the content depends on the class of wheat, genotype, and environmental conditions. The main storage proteins of wheat are the gluten proteins, comprising 80 to 85% of the total wheat proteins, and they are insoluble in water (Van Der Borght et al., 2005). Gluten proteins can be divided into gliadins and glutenins. Gliadins have molecular weights between 30,000 and 80,000, they are single-chained, and they are extremely sticky when hydrated (Van Der Borght et al., 2005). In contrast, glutenins are multi-chained and vary in molecular weight from about 80,000 to several million (Veraverbeke, & Delcour, 2002). In dough formation, gliadins act as plasticizers promoting extensibility, whereas glutenins impart the resistance to extension (Van Der Borght et al., 2005).

2.2. Fusarium Head Blight Disease

The genus *Fusarium* was characterized by Link in 1809, where he included a selection of species known for being devastating plant pathogens that often produced a wide range of secondary metabolites. Agricultural and food safety scientists have shown special interest in some of the produced *Fusarium* metabolites due to their detrimental effects on humans and domesticated animals (Glenn, 2007). Trichothecenes, fumonisins, and zearalenone are the main mycotoxin classes produced by *Fusarium* species. In this regard, *Fusarium graminearum* and *F. culmorum* are the most frequently occurring fungi among the toxin forming *Fusarium* species (Chelkowski, 1998). They are formed in the field before harvest and their occurrence cannot be avoided by plant production minimizing strategies due to the major impact of weather conditions (Döll, & Dänicke, 2011; Glenn, 2007).

The most common fungal agent causing FHB in North America is undoubtedly *F. graminearum* (Goswami, & Kistler, 2004; Shaner, 2003). The primary inoculum for FHB comes from host crop residues on which the fungus overwinters as chlamydospores or saprophytic mycelia; the fungus can also survive on wheat seed. FHB major inoculum source comes from melanized structures, such as perithecia, produced on crop residues. During spring, as temperatures warm up, weather conditions are favorable for the development and maturation of conidia and ascospores produced in perithecia, which often occurs concurrently with cereal crop flowering. When the sticky ascospores are discharged from mature

perithecia, they are spread by wind, insects, and/or splashing water from rain or irrigation to host plants. The deposition of spores on or inside the spike tissue initiates the infection process during wet, warm weather. Wheat spikes (heads) are susceptible from anthesis through the soft dough stage, being at a higher risk of infection during anthesis (stage at which anthers rupture and shed pollen during flowering) (Goswami, & Kistler, 2004; Wegulo, 2012). Initially, the fungus does not penetrate through the epidermis, since the hyphae develop on the exterior surfaces of florets and glumes, allowing fungus to grow toward stomata and other susceptible sites within the inflorescence. In addition, other avenues for direct entry include the base of wheat glumes, where the epidermal and parenchyma cells are thin-walled (Bushnell, Hazen, & Pritsch, 2003). Once inside the floret, the anthers, stigmas and lodicules are most easily colonized. The fungus has a brief biotrophic relationship with its host before switching to the necrotrophic phase, which is associated with an increase in vigor of colonization by the fungus (Goswami, & Kistler, 2004).

2.2.1. *Fusarium* Species

One of the main features of *Fusarium spp.* is that they are well adapted to saprotrophic growth and survival (Kikot, Hours, & Alconada, 2009). Specifically, *F. graminearum*, based on morphological and cultural characteristics, is well recognized for forming abundant homothallic perithecia in a single-spore derived culture. In addition, *F. graminearum* typically causes ear rot of maize and head blight of wheat, barley, and oats in regions with excessive rain and mild temperatures. Likewise, *F. graminearum* produces the trichothecenes nivalenol (NIV) and deoxynivalenol (DON) (plus its derivatives), and the polyketide, which is the most abundant fungal secondary metabolite group, estrogenic metabolite zearalenone (Glenn, 2007; Khaneghah, Martins, von Hertwig, & Bertoldo, 2018). In fact, *F. graminearum* is considered a monophyletic species complex; in other words, it descends from a single ancestor, consisting of nine separate phylogenetic species, some of which are localized on particular continents or geographical areas (O´Donell, Ward, Geiser, Kristler, & Aoki, 2004). Additionally, O´Donell, Kristler, Tacke, & Casper (2000) discovered seven biogeographically structure species within the *F. graminearum* species complex by DNA sequence comparisons. Among this *F. graminearum* species complex, *F. graminearum sensu stricto* is found, which is commonly related to Fusarium head blight (FHB) worldwide (Glenn, 2007; Goswami, & Kistler, 2004). Furthermore, FHB outbreaks in China and other Asiatic regions

are associated with *F. asiaticum* (O´Donell et al., 2000). The rest of the *F. graminearum* species constituting the complex have been isolated from several hosts and they have been studied for their capability of producing FHB (Goswami, & Kistler, 2002).

F. culmorum is morphologically similar to *F. graminearum* since they are closely phylogenetically related (Glenn, 2007). In cool weather areas, *F. culmorum* is likely to be the causal agent of head blight of wheat and other cereals during the growing season (Waalwijk et al., 2004). Moreover, *F. culmorum* is widely distributed in soil, remaining viable for two to four years in the ground in the form of chlamydospores, which are capable of penetrating seeds during germination through lesions in the root or stomas (Scherm et al., 2013). Additionally, the consumption of feed contaminated with both *F. graminearum* and *F. culmorum* has been associated with animal toxicoses, since the two species have similar toxin production capabilities, both producing DON (Glenn, 2007).

2.2.2. Trichothecenes

Trichothecin, isolated from the fungus *Trichothecium roseum*, in 1949 was the first member of this toxin class. Subsequently, *Fusarium* trichothecenes were identified, such as NIV from *Fusarium kyushuense*, T-2 toxin from *F. sporotrichioides*, and DON from *F. graminearum* (Glenn, 2007). At present, trichothecenes comprise a large family of compounds. More than 40 naturally occurring trichothecenes are known from *Fusarium* species, of which diacetoxyscirpenol, T-2 toxin, NIV, and DON are the most important in cereal grains (Desjardins, 2006; Desjardins, & Proctor, 2007).

The inhibition of ribosomal protein synthesis is the major mechanism of trichothecenes toxicity in eukaryotic cells (Desjardins, & Proctor, 2007). Additionally, trichothecenes are phytotoxic to a range of plants where the production of toxins enhances the virulence of some *Fusarium* species on host plants (Desjardins, 2006). Regarding *F. graminearum*, DON production was revealed as a virulence factor enhancing disease development on several host species, including development of FHB and corn ear rot (Desjardins et al., 1996; Proctor, Hohn, & McCormick, 1995).

Fusarium trichothecenes are tricyclic sesquiterpenes (Figure 2.05) that contain a double bond between carbons 9 and 10, and a 12,13-epoxide ring; therefore, they are designated as 12,13-epoxytricothec-9-enes. Other characteristic of *Fusarium* trichothecenes are the various patterns of oxygenation and esterification at positions C-3, C-4, C-7, C-8, and C-15 (Desjardins, & Proctor, 2007).

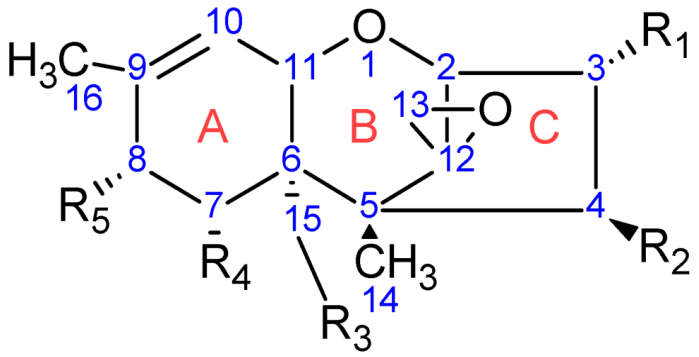

Figure 2.05. Trichothecenes general structure.
(Adapted from Shank, Foroud, Hazendonk, Eudes, & Blackwell, 2011).

The four types of trichothecenes are types A, B, C, and D. Type A and Type B trichothecenes are associated with wheat disease (Foroud, & Eudes, 2009). Type A trichothecene comprises T-2 toxin, and HT-2 toxin synthesized by *F. poae*, *F. langsethiae*, and *F. sporotrichioides*. The most common type of trichothecene is type B, with NIV and DON being synthesized by *F. graminearum* and *F. culmorum* (Khaneghah et al., 2018).

Deoxynivalenol (DON) [(3α,7α)-3,7,15-trihydroxy-12,13-epoxytricothec-9-en-8-one] ($C_{15}H_{20}O_6$) is categorized in the group of trichothecenes, which are sesquiterpenoids containing a central nucleus of hexane cyclic rings and tetrahydropyran (Figure 2.06) (Khaneghah et al., 2018). DON has a molar mass of 296.3 g/mol and crystallizes as colorless needles, which are soluble in water and in polar organic solvents like aqueous solutions of chloroform, ethanol, acetonitrile, methanol, and ethyl acetate (Döll, & Dänicke, 2011; Khaneghah et al., 2018). According to the World Health Organization (2001), DON is stable at 120°C, moderately stable at 180°C, and partially stable at 210°C. Additionally, the International Agency for Research on Cancer (IARC) has classified DON in group 3, so that the mycotoxin is not considered to be carcinogenic to humans (IARC, 1993; Hussein, & Brasel, 2001).

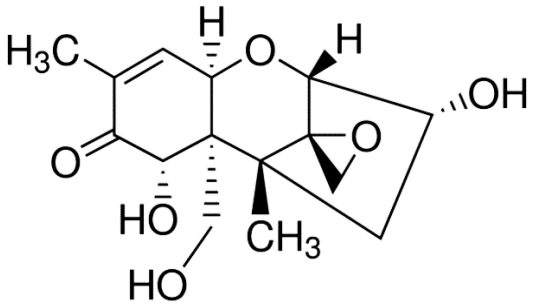

Figure 2.06. Deoxynivalenol (DON) chemical structure.
(Sigma-Aldrich).

Potential impacts of DON in human health may occur after ingestion of contaminated foods from wheat, barley, oats, corn and other small grains. The ingestion of DON contaminated products can result in acute and chronic toxic effects. Regarding humans, the negative effects include short-term nausea, vomiting, diarrhea, abdominal pain, headache, dizziness, and even fever. The strong emetic effect of DON after its consumption is associated to its transportation to the brain, where dopamine receptors in the vomiting center in the medulla are stimulated by DON (Sobrova et al., 2010). In this regard, the provisional maximum tolerable daily ingestion of DON is 1 µg/kg body weight; however, the level to cause acute intoxication was established as 50 µg/kg body weight (JECFA, 2011). Regarding animals, Pestka (2007) proposed a rank of susceptibility to DON ingestion as follows: pigs > mice > rats > poultry ≈ ruminants. The acute symptoms due to DON contaminated feed in animals include feed refusal, and emesis, while chronic dietary exposure to DON caused impaired weight gain, anorexia, decreased nutritional efficiency and immune dysregulation (Bryden, 2012).

The synthesis of DON is induced by an acidic pH, which occurs as a result of the consumption of available nitrogen-containing compounds leading to the formation of ammonium, carrying this condition to the expression of the *Tri5* gene forming a DON precursor (Merhej, Boutigny, Pinson-Gadais, Richard-Forget, & Barreu, 2010). In addition, in an infected plant, DON synthesis prevents the thickening of the cell wall, allowing the fungus to diffuse from an infected branch to a healthy branch through the formation of hyphae (Jansen et al., 2005).

2.2.3. Preventive Measures and Processing Mitigation Strategies

The seemingly ubiquitous occurrence of *Fusarium ssp* creates the need for adequate management of mycotoxin contaminated foodstuffs, such as cereal grains (Döll, & Dänicke, 2011). The amount of DON in wheat-based foodstuff products can correlate with the initial concentration of the mycotoxin in wheat crop, which is mainly influenced by pre-harvest factors like temperature, relative humidity, amount and timing of rainfall, fungal sporulation, wind patterns, and cultural practices (Khaneghah et al., 2018; Glenn, 2007).

2.2.3.1. Agronomic preventative measures

Oldenburg, Valenta, & Sator (2000) ordered the importance of factors for *Fusarium* field infection as follows: weather > tillage > maize as preceding crop > plant protection (fungicide application) > crop

variety. Since weather conditions cannot be manipulated, the first approach to mitigate the mycotoxin production and contamination starts with cultural practices including tillage, crop rotation, fungicide application and variety selection. Historically, moldboard plowing which buried crop residue was commonly done. This was a common cultural method to control various plant diseases as it reduced pathogen survival and inoculum production, which resulted in reduced disease severity and mycotoxin production (Glenn, 2007). Currently, wheat is grown under no-till conditions in order to avoid soil erosion by wind and rain. The lack of tillage has increased dependency on the use of crop rotation and the application of appropriate fungicides to control the severity of FHB and DON synthesis. For example, planting wheat after crops other than maize has been demonstrated to reduce DON concentration by 33% when contrasted with crops planted after maize (Champeil, Doré, & Fourbet, 2004). Furthermore, plant breeding and genetic modification attempt to alter crop cultivars so that they are more resistant to the fungus can contribute to control or lower the DON contamination (Beyer, 2006). The landrace “Sumai 3” is a spring wheat from China that has been utilized as source for FHB resistance in hard red spring wheat, due to its high resistance loci on chromosomes 5A and 3BS (Kumar, Stack, Friesen, & Faris, 2007). However, sources of effective FHB resistance have not been found in adapted durum wheat, resulting in the lack of available resistant durum varieties (Buerstmayr, Ban, & Anderson, 2009).

After wheat harvest, it is likely that levels of DON will remain unaltered if proper conditions are met, such as an a_w less than 0.9 through drying (Khaneghah et al., 2018). *Fusarium* contaminated grains that are thinner, shrunken and lighter than healthy kernels can easily be removed by a cleaning step utilizing gravitational sorting (Webb, & Owens, 2003). In addition, since FHB infected kernels can be pink, optical-based methods can improve the efficiency of cleaning step by evaluating morphological and colorimetric characteristics (Delwiche, Pearson, & Brabec, 2005). However, symptomless kernels with high DON concentrations can occur due to a low disease intensity involving late infection (Wegulo, 2012). Furthermore, blending contaminated grain with uncontaminated grain can achieve the dilution of the mycotoxin concentration below FDA advisory levels (Döll, & Dänicke, 2011).

2.2.3.2. Processing mitigation strategies

Grain infected with *Fusarium* and containing mycotoxin, DON, is greatly discounted at the point of sale. Much of this low value grain enters the animal feed market. For use in human food products,

processing mitigation strategies must be utilized. These strategies include cleaning the grain, milling, and further processing including fermentation, baking, extrusion, cooking, and ozonation (Vidal, Sanchis, Ramos, & Marín, 2016).

More than half of the harvested wheat in the world is subjected to a milling process. Roller milling generally involves the separation of bran and germ from the endosperm. In this regard, the practice of grain conditioning (addition of water pre-milling) does not seem to be a step that aids fungal growth or DON production (Khaneghah et al., 2018). The distribution of the mycotoxin in grain is not uniform, since outer parts (bran and germ) generally have a higher concentration than inner parts (endosperm). The bran layer acts as a physical barrier that restricts the penetration of mycelia into the kernel endosperm. Therefore, bran is the most heavily contaminated fraction with DON (Abbas, Mirocha, Pawlosky, & Pusch, 1985). The distribution of DON in the different fractions obtained from wheat milling relies on the initial fungal penetration (Milani, & Maleki, 2014), subsequently the location of the mycotoxin in the grain will determine if milling will produce flour that has $\leq$ 2 ppm. The deeper the mycotoxin in the kernel the lower the impact of milling on DON removal of specific physical components of the grain and a higher concentration will be found in flour (Young, Fulcher, Hayhoe, Scott, & Dexter, 1984).

The impacts of further processing, such as baking, on DON concentration have been studied (Dexter, Clear, & Preston, 1996; Zhang, & Wang, 2014). Breadmaking involves both fermentation and heat treatment. The fate of DON during fermentation and baking has been studied (Vidal et al., 2014); however, the comparison of different processing methods of bread baking on DON levels remain as a potential study. Bergamini et al., (2010) found that DON concentrations increased when dough was tested immediately before fermentation. Valle-Algarra et al. (2009) reported that DON was stable in dough after fermentation at 30°C. Fermentation temperature might be important. Samar, Neira, Resnik, & Pacin (2001) reported that DON concentration decreased when dough underwent a fermentation of 50°C during 40 min. Regarding baking, it has been hypothesized that reduction of DON levels is a result of the conjugation of the mycotoxin with the food matrix rather than its degradation (Valle-Algarra et al., 2009).

Pasta processing effects on DON levels have also been studied (Brera et al., 2013; Nowicki, Gaba, Dexter, Matsuo, & Clear, 1988; Visconti, Haidukowski, Pascale, & Silvestri, 2004). Of all the steps involving pasta processing, cooking in boiling water had the greatest impact on reducing DON levels due

the water solubility of DON (Visconti et al., 2004). Published studies regarding fate of DON during pasta cooking have reported a 70 to 80% decrease of DON in pasta (Nowicki et al., 1988; Visconti et al., 2004).

2.3. Wheat Dry Milling

Wheat is generally milled to separate the starchy endosperm from the outer layers and germ, and a further reduction of the starchy endosperm so as to obtain flour (common wheat) or semolina (durum wheat) (Pauly et al., 2013). The wheat kernel hardness determines the milling behavior and has to be known by the miller to maximize flour yield. Before milling, kernels are tempered to moisture contents from 15 to 17%, to soften the endosperm and plasticize the bran. The milling process consists of controlled breaking, reduction, and separation stages (Pauly et al., 2013; Posner, 2000). First, grain is passed through a series of corrugated break rolls designed to separate the kernel into its three fractions endosperm, bran, and germ. The coarsely ground endosperm is further reduced by sizing rolls and ultimately through smooth reduction rolls to end-up with flour particles smaller than 180 µm. Milling process is aided by sifters, or sieves, that separate particles based on size, and purifiers that separate particles based on size and weight (Posner, 2000). Roller milling wheat produces four main fractions: flour, bran, germ, and shorts. Shorts are comprised of mill material that is too difficult to separate into flour, bran, and germ.

2.4. Wheat Wet Milling

Approximately 6% of total wheat production is wet milled (Van Der Borght, Goesaert, Veraverbeke, & Delcour, 2005; Sayaslan, Seib, & Chung, 2012). Wet milling separates cereal grains into their main chemical components (protein, starch, lipid, fiber) (Wronkowska, 2016).

Wet milling wheat begins with flour or semolina produced by dry milling since bran becomes entangled with gluten proteins when wet milling whole wheat kernel. Thus, the germ (lipid) and bran (fiber) fractions have been removed by dry milling operation. The Martin (dough-washing), the Batter, the Alfa-Laval/Raisio, the Hydrocyclone, and the High-Pressure Disintegration (HD) processes are the five commercial wet milling methods that start with wheat flour rather than wheat kernels (Sayaslan, 2004).

Wet milling wheat flour results in the separation and purification of gluten proteins and starch granules based on differences in their aggregation, particle sizes, and sedimentation rates (Sayaslan, 2004; Van Der Borght et al., 2005). Native gluten proteins are able to build a three-dimensional network

when the flour is mixed with water. Initially, the starch granules are embedded in the matrix. Starch density is 1.4 g/cm^3 and wet gluten density is 1.1 g/cm^3. This difference in density is used to separate them from the dough with centrifugation (Meuser, 1994).

2.4.1. Wet Milling Products

2.4.1.1. Wheat starch

Commercial prime A- starch isolated from wheat flour is composed on a dry basis primarily of starch (>98%) with low levels of lipids (0.8%), proteins (<0.3%), and minerals (0.1-0.3%) (Sayaslan, 2004). Wheat starch is used to produce modified starches, such as acid-thinned, bleached, oxidized, cross-linked, substituted, and other modified starches, which are used in food and non-food applications (Maningat, & Seib, 1997). Additionally, wheat starch can be converted to hydrolyzed starch products like sweeteners, particularly in Europe and Australia (Sayaslan, 2004).

2.4.1.2. Vital wheat gluten

Vital wheat gluten is a valuable co-product of a wet milling process that yields wheat starch (Maningat, & Bassi, 1999). According to the Codex Standard for Wheat Protein Products, wheat gluten on a dry basis must contain 73% protein (%Nx5.7), and must not exceed 10% moisture, 2% ash, 2% free lipids, and 1.5% fiber contents (FAO, 2001). Typically, commercial wheat gluten has 10% moisture, up to 82% protein (%Nx5.7), 2% free lipids, 1.5% ash, and ~1% fiber. Most of the vital gluten is produced and consumed in North America, the European Union, and Australia (Sayaslan, 2004). In the US, much of the vital wheat gluten is used to strengthen frozen dough products and specialty baked goods. (Holcomb, 1999). In addition, vital wheat gluten is used in breakfast cereals, snacks, meat and cheese analogs, breading and batter mixes, pizza, and in meat, fish and poultry products (Bergthaller, 1997).

2.4.2. Wheat and Wheat Flour Specifications for Wet Milling

Straight grade flour, obtained from a conventional dry milling, and high-extraction coarse flour, obtained from a short flow dry milling, are used in wet milling processes (Sayaslan, 2004). For wet milling, flour from hard wheats is typically used in North America; whereas soft wheat flour is preferred in Europe (Maningat, & Bassi, 1999). Flours used in the process do not have to be bleached, malted or enriched, and it is preferable that they have more than 11% protein content, low starch damage, low ash content, and low α-amylase activity (Lindhauer, & Bergthaller, 2002).

2.4.3. Flour Fractionation

Kempf, & Röhrmann (1989) list fifteen wet milling processes starting from wheat kernels or flour. However, only five of those processes have been used industrially, and all of them start with flour. Currently in North America the Martin, Alfa-Laval/Raisio, and Hydrocyclone processes are commercially used, while the High-Pressure Disintegration (HD) is used in Europe (Sayaslan, 2004). Between 1940 and 1960, the Batter process was used but it is now discontinued (Maningat, & Bassi, 1999).

2.4.3.1. Martin process

In 1745, Beccari, an Italian chemist, reported a dough washing process, which was further developed and proposed by Martin in Paris in 1853, making it the oldest wet milling process to isolate wheat starch and vital wheat gluten (Maningat, & Bassi, 1999). The Martin process was the only commercial process for the production of wheat starch until the 20th century, and even up to the 1970s it remained the most popular wet milling process for wheat flour (Sayaslan, 2004). The Martin process underwent some improvements over time, such as the conservation of fresh water through a water recycling process and the development of new equipment for efficient starch and gluten separation (Maningat, & Bassi, 1999).

The process begins with the formation of a stiff dough (40 to 60% of water), which is allowed to rest in order to let the gluten proteins fully hydrate. Then the dough is washed in either roller bars under a spray of water, or in a continuous kneader with additional water to remove the starch and water extractable fractions from the rubbery mass of gluten. The starch suspension is passed through screens to remove any gluten residue. The extracted gluten is further kneaded with excess water to wash any remaining starch, then dewatered, and flash dried resulting in vital wheat gluten. The starch slurry is purified by further sieving, centrifuging, and the use of hydrocyclones (Sayaslan, 2004; Van Der Borght et al., 2005). Approximately 85% of starch is recovered as A-starch fraction, while around 85% of protein content can be recovered as the vital gluten fraction (Maningat, & Bassi, 1999). On a laboratory scale, the Martin process can be mimic following the gluten index procedure as described by AACCI Approved Method 38-12.02.

2.4.3.2. Batter process

The Batter process was industrially used in North America and Australia between 1940s and 1950s (Van Der Borght et al., 2005). The process begins with the formation of a batter by mixing equal amounts of flour and warm water (55°C). The batter is aged for 30 minutes, followed by the addition of cold water equal to two times the weight of the flour added. By further mixing, curd-like gluten aggregates are produced, while the starch is washed away using screens (Sayaslan, 2004; Van Der Borght et al., 2005). Purification of protein and starch fractions is similar to the one done in the Martin process. Total starch recovery has been stated as high as 90%, and up to 85% of protein recovery (Sayaslan, 2004).

2.4.3.3. Hydrocyclone process

This process is considered new among the existing wet milling processes. It was developed in Holland by the KSH Company during the 1970's. It has some advantages such as lower cost compared to the existing processes, a wide range of operating conditions, and reduced water utilization (Maningat, & Bassi, 1999). The process involves the development of dough, followed by the formation of a dough-water dispersion by adding water under shear-mixing conditions, which is further pumped into a series of hydrocyclones that ends with the precipitation of gluten proteins (Maningat, & Bassi, 1999; Sayaslan, 2004). After separating the starch from the gluten fraction, the starch is washed with countercurrent water to remove impurities, and then passed through a hydrocyclone to concentrate it for its final flash-dry (Maningat, & Bassi, 1999). Approximate yields of the recovered fractions using this process were not reported in literature. On a laboratory scale, the Hydrocyclone process can be reproduced with medium sheared dough-water dispersion methods.

2.4.3.4. Alfa-Laval/Raisio process

In 1976, the Alfa-Laval/Raisio plant, located in Finland, essentially developed a variation from a previous wet milling process named Fesca. The Fesca process is a direct centrifugation process, where wheat flour is mixed with water and centrifuged to produce micrometer-sized gluten agglomerates that are separated from starch by centrifugal forces (Van Der Borght et al., 2005). The variation from the Alfa-Laval/Raisio to the Fesca process was to use warm water (45°C) to form a batter, which was then aged, and finally centrifuged in a process held at 50°C to maintain gluten vitality (Sayaslan, 2004). This process

can remove about 80% of vital wheat gluten from the flour (Maningat, & Bassi, 1999). Approximate starch yields were not reported in literature.

2.4.3.5. High-Pressure Disintegration (HD) process

This process was developed by the Technical University of Berlin in collaboration with Westfalia Separator AG of Germany, designed originally to extract starch from potato and was later adapted for wheat flour wet milling in 1984 (Witt, 1997). The process starts with a rapid mixing of flour and water by a high-pressure homogenizer in order to dislodge the starch from the gluten matrix and disperse both starch granules and gluten fibrils through the liquid phase. More water is then added and the slurry is sent to a decanter-centrifuge to separate it in three phases: 1) A-starch, 2) gluten entangled with B-starch, and 3) fiber. The aid of centrifuge forces and screen washings are needed for further fraction purification (Sayaslan, 2004). A-starch recovery ranges from 80 to 85%, while the vital wheat gluten recovery is up to 85% (Witt, 1997). On a laboratory scale, the Alfa-Laval/Raisio process, as well as the HD process, can be reproduced with highly sheared flour-water dispersion methods.

2.5. References

Abbas, H. K., Mirocha, C. J., Pawlosky, R. J., & Pusch, D. J. (1985). Effect of cleaning, milling, and baking on deoxynivalenol in wheat. *Applied and Environmental Microbiology*, *50*, 482-486.

Apprich, S., Tirpanalan, O., Hell, J., Reisinger, M., Bohmdorfer, S., Siebenhandl-Ehn, S., Novalin, S., & Kneifel, W. (2014). Wheat bran-based biorefinery 2: Valorization of products. *Food Science and Technology*, *56*, 222-231.

Bergamini, E., Catellani, D., Dall'asta, C., Galaverna, G., Dossena, A., & Marchelli, R. (2010). Fate of Fusarium mycotoxins in the cereal product supply chain: The deoxynivalenol (DON) case within industrial breadmaking technology. *Food Additives & Contaminants: Part A*, *27*, 677-687.

Bergthaller, W. J. (1997). New uses of wheat gluten and non-starch wheat components. In J. L. Steele, and O. K. Chung (Eds.), *Proc. Int. Wheat Quality Conference* (pp. 285-301). Manhattan, KS: Grain Industry Alliance.

Beyer, M., Klix, M. B., Klink, H., & Verreet, J. A. (2006). Quantifying the effects of previous crop, tillage, cultivar, and triazole fungicides on the deoxynivalenol content of wheat grain –a review. *Journal of Plant Diseases and Protection*, *113*, 241-246.

Brandolini, A., & Hidalgo, A. (2012). Wheat germ: not only a by-product. *International Journal of Food Sciences and Nutrition*, *63*, 71-74.

Brera, C., Peduto, A., Debegnach, F., Pannunzi, E., Prantera, E., Gregori, E., De Giacomo, M., & De Santis, B. (2013). Study of the influence of the milling process on the distribution of deoxynivalenol content from the caryopsis to cooked pasta. *Food Control*, *32*, 309-312.

Bryden, W. L. (2012). Mycotoxin contamination of the feed supply chain: Implications for animal productivity and feed security. *Animal Feed Science and Technology*, *173*, 134-158.

Bushnell, W. R., Hazen, B. E., & Pritsch, C. (2003). Histology and physiology of Fusarium head blight. In K. J. Leonard, and W. R. Bushnell (Eds.), *Fusarium head blight of wheat and barley* (pp. 44-83). Minneapolis, MN: APS Press.

Buerstmayr, H., Ban, T., & Anderson, J. A. (2009). QTL mapping and marker-assisted selection for Fusarium head blight resistance in wheat (review). *Plant Breeding*, *128*, 1-26.

Champeil, A., Doré, T., & Fourbet, J. F. (2004). Fusarium head blight: epidemiological origin of the effects of cultural practices on head blight attacks and the production of mycotoxins by Fusarium in wheat grains. *Plant Science*, *166*, 1389-1415.

Chelkowski, J. (1998). Distribution of Fusarium species and their mycotoxins in cereal grains. In K.K. Sinha, and D. Bhatnagar (Eds.), *Mycotoxins in agriculture and food safety* (pp. 45-64). New-York-Basel-Hong Kong: Marcel Dekker, Inc.

Cornell, H. J. (2012). The chemistry and biochemistry of wheat. In S. P. Cauvain (Ed.), *Breadmaking: Improving quality* (pp. 35-76). Cambridge, UK: Woodhead Publishing Limited.

De Vries, A. PH. (1971). Flowering biology of wheat, particularly in view of hybrid seed production – A review. *Euphytica*, *20*, 152-170.

Delwiche, S. R., Pearson, T. C., & Brabec, D. L. (2005). High-speed optical sorting of soft wheat for reduction of deoxynivalenol. *Plant Disease*, *89*, 1214-1219.

Desjardins, A. E. (2006). *Fusarium mycotoxins. Chemistry, genetics, and biology*. (p. 268). Minneapolis, MN: American Phytopathological Society Press.

Desjardins, A. E., & Proctor, R. H. (2007). Molecular biology of Fusarium mycotoxins. *International Journal of Food Microbiology*, *119*, 47-50.

Desjardins, A. E., Proctor, R. H., Bai, G., McCormick, S. P., Shaner, G., Buechely, G., & Hohn, T. M. (1996). Reduced virulence of trichotecene-nonproducing mutants of Gibberella zeae in wheat field test. *Molecular Plant-Microbe Interactions*, *9*, 775-781.

Dexter, J. E., Clear, R. M., & Preston, K. R. (1996). Fusarium head blight: Effect on the milling and baking of some Canadian wheats. *Cereal Chemistry*, *73*, 695-701.

Döll, S., & Dänicke, S. (2011). The Fusarium toxins deoxynivalenol (DON) and zearalenone (ZON) in animal feeding. *Preventive Veterinary Medicine*, *102*, 132-145.

FAO. (2001). Codex standard for wheat gluten. *Codex Standard*, 163-1987. Rome: Food and Agriculture Organization.

FAO. (2018). Food and Agriculture Organization Statistics. On-line: http://www.fao.org/faostat/en/#data/QC. Food and Agriculture Organization: Rome.

Foroud, N. A., & Eude, F. (2009). Trichothecenes in cereal grains. *International Journal of Molecular Sciences*, *10*, 147-173.

Ghafoor, K., Ozcan, M. M., Al-Juhaimi, F., Babiker, E. E., Sarker, Z. I., Ahmed, I. A. M., & Ahmed, M. A. (2017). Nutritional composition, extraction, and utilization of wheat germ oil: A review. *European Journal of Lipid Science and Technology*, *119*, 1-9.

Glenn, A. E. (2007). Mycotoxigenic Fusarium species in animal feed. *Animal Feed Science and Technology*, *137*, 213-240.

Goswami, R. S., & Kistler, H. C. (2002). Assessment of the differential ability of Fusarium strains to spread on wheat and rice. *2002 National Fusarium Head Blight Forum Proceedings*. East Lansing: Michigan State University (p.163).

Goswami, R. S., & Kistler, H. C. (2004). Heading for disaster: Fusarium graminearum on cereal crops. *Molecular Plant Pathology*, *5*, 515-525.

Holcomb, R. B. (1999). Overview of domestic wheat gluten industry. WF-559. OSU extension facts. Stillwater, OK: Oklahoma State University Cooperative Extension Service.

Hussein, H. S., & Brasel, J. M. (2001). Toxicity, metabolism, and impact of mycotoxins on humans and animals. *Toxicology*, *167*, 101-134.

International Agency for Research on Cancer (IARC). (1993). IARC monographs on the evaluation of carcinogenic risks to humans. *WHO*, *56*, 397-444.

Jansen, C., von Wettstein, D., Schäfer, W., Kogel, K., Felk, A., & Maier, F. J. (2005). Infection pattern in barley and wheat spikes inoculated with wild-type and trichodiene synthase gene disrupted Fusarium graminearum. *Proceedings of the National Academy of Sciences of the United States of America*, *102*, 16892-16897.

JECFA (Joint FAO/WHO Expert Committee on Food Additives). (2011). *WHO Technical report series*, *959*, 37-48.

Kempf, W., & Röhrmann, C. (1989). Process for the industrial production of wheat starch form whole wheat. In Y. Pomeranz (Ed.), *Wheat is Unique: Structure, Composition, Processing, End-use Properties, and Products* (pp. 521-540). Minneapolis, MN: AACC International.

Khaneghah, A. M., Martins, L. M., von Hertwig, A. M., & Bertoldo, R. (2018). Deoxynivalenol and its masked forms: Characteristics, incidence, control and fate during wheat and wheat based products processing – A review. *Trends in Food Science & Technology*, *71*, 13-24.

Kikot, G. E., Hours, R. A., & Alconada, T. M. (2009). Contribution of cell wall degrading enzymes to pathogenesis of Fusarium graminearum: A review. *Journal of Basic Microbiology*, *49*, 231–241.

Koehler, P., & Wieser, H. (2013). Chemistry of Cereal Grains. In M. Gobbetti, and M. Gänzle (Eds.), *Handbook on Sourdough Biotechnology* (pp. 11-45). New York, NY: Springer.

Kumar, S., Stack, R. W., Friesen, T. L., & Faris, D. J. (2007). Identification of a novel Fusarium head blight resistance quantitative trait locus on chromosome 7A in tetraploid wheat. *Phytopathology*, *97*, 592-597.

Lempereur, I., Rouau, X., & Abecassis, J. (1997). Genetic and agronomic variation in arabinoxylan and ferulic acid contents of durum wheat (Triticum durum L.) grain and its milling fractions. *Journal of Cereal Science*, *25*, 103-110.

Lersten, N. R. (1987). Morphology and anatomy of the wheat plant. In E. G. Heyne (Ed.), *Wheat and wheat improvement, Agronomy Monograph 13* (pp. 33-74). Madison, WI: ASA, CSSA, SSSA.

Lindhauer, M. G., & Bergthaller, W. P. (2002). Characteristics for the assessment of starch extractability from wheat. In V. P. Yuryev, A. Cesaro, and W. J. Bergthaller (Eds.), *Starch and starch*

containing origins: structures, properties and new technologies (pp. 375-382). New York, NY: Novo Science Publishers.

Maningat, C. C., & Bassi, S. D. (1999). Wheat starch production. In M. Tumbleson, P. Yang, and S. Eckhoff (Eds.), *Proc. Int. Starch Technology Conference* (pp. 26–40) Urbana, IL: University of Illinois.

Maningat, C. C., & Seib, P. A. (1997). Update on wheat starch and its use. In J. L. Steele, and O. K. Chung (Eds.), *Proc. Int. Wheat Quality Conference* (pp. 261–284). Manhattan, KS: Grain Industry Alliance.

Matsuo, R. R. (1994). Durum wheat: its unique pasta-making properties. In W. Bushuk and V. F. Rasper (Eds.), Wheat production properties and quality (pp. 169-178). UK: Chapman & Hall.

Merhej, J., Boutigny, A. L., Pinson-Gadais, L., Richard-Forget, F., & Barreau, C. (2010). Acidic pH as a determinant of TRI gene expression and trichothecene B biosynthesis in Fusarium graminearum. *Food Additives & Contaminants: Part A*, *27*, 710-717.

Meuser, F. 1994. Wheat utilization for the production of starch, gluten and extruded products. In W. Bushuk, and V.F. Rasper (Eds.), *Wheat production, properties and quality* (pp. 179-204). UK: Cambridge University Press.

Milani, L., & Maleki, G. (2014). Effects of processing on mycotoxin stability in cereals. *Journal of the Science of Food and Agriculture*, *94*, 2372-2375.

Nowicki, T. W., Gaba, D. G., Dexter, J. E., Matsuo, R. R., & Clear, R. M. (1988). Retention of the Fusarium mycotoxin deoxynivalenol in wheat during processing and cooking of spaghetti and noodles. *Journal of Cereal Science*, *8*, 198-202.

O´Donell, K., Kristler, H. C., Tacke, B. K., & Casper, H. H. (2000). Gene genealogies reveal global phylogeographic structure and reproductive isolation among lineages of Fusarium graminearum, the fungus causing wheat scab. *Proceedings of the National Academy of Sciences of the United States of America*, *97*, 7905-7910.

O´Donell, K., Ward, T. J., Geiser, D. M., Kristler, H. C., & Aoki, T. (2004). Genealogical concordance between the mating type locus and seven other nuclear genes supports formal recognition of nine

phylogenically distinct species within Fusarium graminearum clade. *Fungal Genetics and Biology*, *41*, 600-623.

Oleson, B. T. (1994). World wheat production, utilization and trade. In W. Bushuk and V. F. Rasper (Eds.), Wheat production properties and quality (pp. 1-11). UK: Chapman & Hall.

Oldenburg, E., Valenta, H., & Sator, C. 2000. Risk assessment and avoidance strategies for feed production. In S. Dänicke, and E. Oldenburg (Eds.), *Risk factors for Fusarium toxin production and avoidance strategies for feed production and feeding* (pp. 5-34). Germany: Landbauforsch. Völk. SH 216.

Pask, A. (2012). Determining key developmental stages. In: D. Mullan and M. Reynolds (Eds.), *Physiological breeding II: A field guide to wheat phenotyping* (pp. 72-77). Mexico City: CIMMYT.

Pauly, A., Pareyt, B., Fierens, E., & Delcour, J. A. (2013). Wheat (Triticum aestivum L. and turgidum L. ssp. durum) kernel hardness: II. Implications for end-product quality and role of puroindolines therein. *Comprehensive Reviews in Food Science and Food Safety*, *12*, 427-438.

Peña, R. J. (2002). Wheat for bread and other foods. In B. C. Curtis, S. Rajaram, and H. Gómez Macpherson (Eds.), *Bread Wheat* (pp. 554). Rome: FAO.

Percival, J. (1921). The inflorescence. In *The wheat plant A monograph* (pp. 100-109). London: Duckworth and Co.

Pestka, J. J. (2007). Deoxynivalenol: Toxicity, mechanisms and animal health risks. *Animal Feed Science and Technology*, *137*, 283-298.

Pomeranz, Y. (1982). Grain structure and end-use properties. *Food Microstructure*, *1*, 107-124.

Pomeranz, Y. (1988). Chemical composition of kernel structures. In Y. Pomeranz (Ed.), *Wheat: Chemistry and technology volume 1* (pp. 97-158). St. Paul, MN: AACCI.

Posner, E. S. (2000). Wheat. In K. Kulp (Ed.), *Handbook of cereal science and technology* (pp. 1-30). US: CRC Press.

Proctor, R. H., Hohn, T. M., & McCormick, S. P. (1995). Reduced virulence of Gibberella zeae caused by disruption of a trichothecene toxic biosynthetic gene. *Molecular Plant-Microbe Interactions*, *8*, 593-601.

Ranhotra, G. S. (1994). Wheat: contribution to world food supply and human nutrition. In W. Bushuk and V. F. Rasper (Eds.), Wheat production properties and quality (pp. 12-24). UK: Chapman & Hall.

Samar, M. M., Neira, M. S., Resnik, S. L., & Pacin, A. (2001). Effect of fermentation on naturally occurring deoxynivalenol (DON) in Argentinean bread processing technology. *Food Additives & Contaminants*, *18*, 1004-1010.

Sayaslan, A. (2004). Wet-milling of wheat flour: Industrial processes and small-scale test methods. *Journal of Food Science and Technology*, *37*, 499-515.

Sayaslan, A., Seib, P. A., & Chung, O. K. (2012). A bench-scale high-shear wet-milling test for wheat flour. *Journal of Food Engineering*, *11*, 305-317.

Scherm, B., Balmas, V., Spanu, F., Pani, G., Delogu, G., & Pasquali, M. (2013). Fusarium culmorum: Causal agent of foot and root rot and head blight on wheat. *Molecular Plant Pathology*, *14*, 323-341.

Shaner, G. E. (2003). Epidemiology of Fusarium head blight of small grain cereals in North America. In K. J. Leonard, and W. R. Bushnell (Eds.), *Fusarium Head Blight of wheat and barley* (pp. 84-199). Minneapolis, MN: American Phytopathological Society.

Shank, R. A., Foroud, N. A., Hazendonk, P., Eudes, F., & Blackwell, B. A. (2011). Current and future experimental strategies for structural analysis of trichotecene mycotoxin – A prospectus. *Toxins*, *3*, 1518-1553.

Simmons, S. R., Oelke, E. A., & Anderson, P. M. (1985). Growth and development guide for spring wheat. University of Minnesota Agricultural Extension Folder AG-FO-2547.

Sobrova, P., Adam, V., Vasatkova, A., Beklova, M., Zeman, L., & Kizek, R. 2010. Deoxynivalenol and its toxicity. *Interdisciplinary Toxicology*, *3*, 94-99.

Sramková, Z., Gregová, E., & Sturdík, E. (2009). Chemical composition and nutritional quality of wheat grain. *Acta Chimica Slovaca*, *2*, 115-138.

Valle-Algarra, F. M., Mateo, E. M., Medina, A., Mateo, F., Gimeno-Adelantado, J. V., & Jimenez, M. (2009). Changes in ochratoxin A and type B trichothecenes contained in wheat flour dough fermentation and bread-baking. *Food Additives & Contaminants*, *26*, 896-906.

Van Der Borght, A., Goesaert, H., Veraverbeke, W. S., & Delcour, J. A. (2005). Fractionation of wheat and wheat flour into starch and gluten: overview of the main processes and the factors involved. *Journal of Cereal Science*, *41*, 221-237.

Veraverbeke, W. S., & Delcour, J. A. (2002). Wheat protein composition and properties of wheat glutenin in relation to breadmaking functionality. *Critical Reviews in Food Science and Nutrition*, *42*, 179–208.

Vidal, A., Marín, S., Morales, H., Ramos, A.J., & Sanchis, V. (2014). The fate of deoxynivalenol and ochratoxin A during the breadmaking process, effects of sourdough use and bran content. *Food and Chemical Toxicology*, *68*, 53-60.

Vidal, A., Sanchis, V., Ramos, A. J., & Marín, S. (2016). The fate of deoxynivalenol through wheat processing to food products. *Food Science*, *11*, 34-39.

Visconti, A., Haidukowski, E. M., Pascale, M., & Silvestri, M. (2004). Reduction of deoxynivalenol during durum wheat processing and spaghetti cooking. *Toxicology Letters*, *153*, 181-189.

Waalwijk, C., Van Der Heide, R., De Vries, I., Van Der Lee, T., Schoen, C., Costrel-de Corainville, G., Häuser-Hahn, I., Kastelein, P., Köhl, J., Lonnet, P., Demarquet, T., & Kerma, G. H. J. (2004). Quantitative detection of Fusarium species in wheat using TaqMaq. *European Journal of Plant Pathology*, *110*, 481-494.

Webb, C., & Owens, G. W. (2003). Milling and flour quality. In S. P. Cauvain (Ed.), *Woodhead publishing series in food science, technology and nutrition* (pp. 200-219). UK: Woodhead Publishing.

Wegulo, S. N. (2012). Factors influencing Deoxynivalenol accumulation in small grain cereals. *Toxins*, *4*, 1157-1180.

Witt, W. 1997. Modern method of separating the components of wheat. In J. L. Steele, and O. K. Chung (Eds.), *Proc. Int. Wheat Quality Conference* (pp. 231–248). Manhattan, KS: Grain Industry Alliance.

World Health Organization (WHO). (2001). Deoxynivalenol. In: *Food additives series 47*. Food and nutrition paper 74. International program on chemical safety (pp. 419-556). Switzerland: World Health Organization.

Wronkowska, M. (2016). Wet-milling of cereals. *Journal of Food Processing and Preservation*, *40*, 572-580.

Young, J. C., Fulcher, R. G., Hayhoe, J. H., Scott, P. M., & Dexter, J. E. (1984). Effect of milling and baking on deoxynivalenol content of eastern Canadian wheat. *Journal of Agricultural and Food Chemistry*, *32*, 659-664.

Zhang, H., & Wang, B. (2014). Fate of deoxynivalenol and deoxynivalenol-3-glucoside during wheat milling and Chinese steamed bread processing. *Food Control*, *44*, 86-91.

3. WET MILLING TECHNIQUE APPLIED TO DEOXYNIVALENOL CONTAMINATED WHEAT DRY MILLED FRACTIONS

3.1. Abstract

The consumption of wheat contaminated with deoxynivalenol (DON), a highly water-soluble *Fusarium* mycotoxin, represents a health threat to animals and humans. Wheat is usually dry milled into flour, bran, germ and shorts. The aim of this research was to determine the extent of DON removal from these wheat fractions during wet milling using the Martin process. Wet milling involves the use of water to separate starch, protein, lipid, and fiber. After wet milling the farina/semolina, shorts, and bran, most of the mycotoxin was concentrated in the freeze-dried water soluble fraction. On average, more than 90% of the mycotoxin was accounted from the wet milling. After wet milling of farina/semolina, gluten extracted from hard red spring wheat (HRSW) contained low levels of DON <0.60 mg/kg, while gluten extracted from durum wheat (DW) contained no detectable DON. The remainder of DON was found in the water soluble fraction. After wet milling shorts, DON levels were only detected in the freeze-dried water soluble fraction. After wet milling the bran fraction, DON distribution in the isolated starch was 3% and 5% in HRSW and DW, respectively; in de-starched bran was 7% and 1% in HRSW and DW, respectively; and in freeze-dried water solubles fraction was 83% and 88% in HRSW and DW, respectively. Results indicated that wet milling was effective in removing DON from the studied dry milled fractions. The study suggests the implementation of the wet milling technique could be useful in reducing or eliminating DON from dry milling products, which would allow them to be used in animal and human food.

3.2. Introduction

Fusarium spp. are the casual organism for seed disease commonly referred to as Fusarium Head Blight (FHB) in small grains (Döll, & Dänicke, 2011). *Fusarium* spores land on cereal inflorescences such as wheat spike. Hyphae from germinating spores penetrate into the developing kernel and release hydrolases that degrade protein and starch (Kang, & Buchenauer, 2002). The hyphae absorb the resulting amino acids and sugars where they are used as energy source and building blocks for macromolecules associated with *Fusarium* growth and development. *Fusarium* can infect the grain from anthesis to physiological maturity (Champeil, Doré, & Foubert, 2004). The longer the grain is infected, the greater is the negative effect on grain quality, particularly test weight and kernel weight.

The destructive disease FHB has been estimated to have caused $1.554 billion in revenue loss for durum and hard red spring wheat grown in North Dakota from 2000 to 2014 (Wilson, McKee, Nganje, Dahl, & Bangsund, 2017). Economic loss is due to reduction in grain yield and quality and to the presence of the mycotoxin, Deoxynivalenol (DON). Reduction in test weight, kernel weight, and kernel size are associated with reduced milling yield, all of which reduces the value of the crop. Value is also reduced by the presence of DON, a mycotoxin produced by *Fusarium*.

DON can be found in the bran, germ and endosperm layers. *Fusarium* produces DON in grain as a response to the host's plant defense pathway. DON synthesis enhances the production of H_2O_2 by the host plant, which causes cell death and leads to the *Fusarium* necrotrophic infection, accompanied by more DON synthesis (Audenaert, Vanheule, Hofte, & Haesaert, 2013). However, mycotoxin contamination levels are highly variable since the *Fusarium* infection relies on several other factors, such as different regions, years, weather conditions, cultivars, and sowing times (Cheli, Pinotti, Rossi, & Dell'Orto, 2013).

DON belongs to the structural group of type B trichothecenes, characterized by tricyclic 12,13-epoxytrichothec-9-ene core structure. DON, a colorless crystal, has a molar mass of 296.3 g/mol, and is highly soluble in water, as well as in polar organic solvents, like chloroform, ethanol, and acetonitrile (Audenaert, Vanheule, Hofte, & Haesaert, 2013; Khaneghah, Martins, von Hertwig, & Bertolo, 2018).

Food safety concerns regarding human and livestock toxicosis caused by DON has caused the FDA to establish advisory levels for humans (1 mg/kg in finished wheat products), ruminants (10 mg/kg), and swine (5 mg/kg) (Wegulo, 2012). Some of the recorded symptoms in humans due to consumption of DON contaminated grain are abdominal pain, nausea, vomiting, headache, diarrhea, and fever (Sobrova et al., 2010). In addition, animal toxicosis manifests as feed refusal, slow growth, intestinal hemorrhage, and suppression of immune responses (Petska, 2007).

Human and animal toxicity of DON and the associated decline in grain value have driven research aimed at finding ways to reduce or eliminate DON from grain or grain products. Density segregation for grain selection can effectively remove kernels with high DON levels due to the low relative density of scabby kernels (Scott, Kanhere, Lau, Dexter, & Greenhalgh, 1983; Seitz, Yamazaki, Clements, Mohr, & Andrews, 1985). However, the severity of FHB is not necessarily correlated with the level of DON

contamination in grains since DON can occur in visually uninfected kernels (Seitz, & Bechtel,1985). DON is unevenly distributed in grain and typically accumulates greatest in bran, intermediate in germ and lowest in endosperm (Vidal, Sanchis, Ramos, & Marín, 2016). Milling does not destroy or remove DON from the grain but physically separates the bran and germ from the endosperm (Kaushik, 2015; Kushiro, 2008; Wu, Kuca, Humpf, Klimova, & Cramer, 2017). Therefore, if the amount of DON in the endosperm is less than or equal to 1 mg/kg, the endosperm can be further milled into semolina or flour and be used as ingredients in human food products. To illustrate this, the reports from Lancova et al. (2008) and Gärtner, Munich, Kleijer, & Mascher (2008), both utilizing an automatic laboratory Bühler mill (MLU-202), showed that the highest concentrations of DON after milling were found in the bran, while the lowest concentrations were in the reduction flours, still denoting the presence of the mycotoxin in each of the dry milled fractions.

In addition, research has been conducted to determine the effects of processing on DON stability in food products. De Angelis, Monaci, Pascales, & Visconti (2013) and Vidal, Morales, Sanchis, Ramos, & Marín (2014) reported that DON did not decompose during bread baking. DON has been reported to be stable at temperatures up to 120°C (Döll, & Dänicke, 2011). DON is highly water soluble (Sobrova et al., 2010). Cooking spaghetti in large amounts of boiling water can transfer DON from the dough to the cooking water (Kushiro, 2008; Wu et al., 2017). Visconti, Haidukowski, Pascale, & Silvestri (2004) reported that 60 to 75% of DON in dry pasta leached from the pasta into the cooking water.

The high water solubility of DON and the documented decline of DON in cooked (boiled) pasta suggest that DON could be extracted into water during wet milling. Research on the fate of DON and other mycotoxins, like nivalenol (NIV) and zearalenone (ZEN), during the wet milling process has been reported on corn (Bennett, & Anderson, 1978; Lauren, & Ringrose, 1997). These authors reported that during wet milling, the highly water soluble mycotoxins DON and NIV were essentially concentrated in residual water and were found at very low levels in solids like germ, fiber, gluten, and starch; whereas the relatively insoluble ZEN was found in higher concentration in the solid compared to water fractions.

Wet milling process involves separation and purification based on chemistry. Protein, starch, lipid, and fiber are common products or fractions of wet milled grain. Vital wheat gluten and wheat starch are commercially produced by wheat wet milling. Wheat wet milling usually begins with the refined flour

produced during a dry milling process (Sayaslan, 2004; Van Der Borght, Goesaert, Veraverbeke, & Delcour, 2005). Basically, the traditional dough-washing wet milling process or Martin process involves developing dough by mixing flour with water. The dough is washed with a large volume of water which removes starch and water soluble compounds from the gluten matrix. The starch and water soluble material are separated by centrifugation (Maningat, Seib, Bassi, Woo, & Lasater, 2009).

Information is limited concerning the effectiveness of wet milling processes in removing DON from contaminated wheat dry milling fractions (farina/semolina, shorts, bran). Removal of DON would result in added value to low value contaminated grain. In this regard, the aim of the research was to determine the extent of DON removal from wheat fractions during wet milling using the Martin process.

3.3. Materials and Methods

3.3.1. Materials

HRSW and DW samples were selected based on their DON content. The control samples contained no detectable DON and three samples each of HRSW and DW were selected because they contained more than 2 mg/kg DON. DW samples were obtained in 2015 and 2016. They were collected as part of the annual durum crop quality survey by the Montana and North Dakota National Agricultural Statistics Service offices. Durum wheat samples, approximately 0.75 to 1.5 kg, were collected during the harvest season; samples came directly from grower fields, farm bins, and local elevators. Samples were stored in sealed moisture-proof plastic bags. Two durum samples from North Dakota collected in 2015, one sample from North Dakota collected in 2016, and one sample from Montana collected in 2015 were included in the study. HRSW experimental lines ND826, NDHRS16-12-42, and NDHRS16-12-46 were grown in 2016 at eastern ND Red River Valley testing sites and the cultivar WB Mayville was grown at Williston, ND. All HRSW samples were kindly provided by the NDSU Hard Spring Wheat breeding program. Each sample was cleaned by passing through a Carter – Day dockage tester and divided into four 250 g subsamples using a Boerner sample divider and stored at -18°C until needed.

3.3.2. Grain and Farina/Semolina Proximate Analysis

Grain moisture content, ash content, protein content, total starch, and falling number were determined using AACCI Approved Methods 44-15.02, 08-01.01, 46-30.01, 76-13.01, and 56-81.03, respectively. Additionally, 1000-kernel weight was determined by removing all dockage, shrunken and

broken kernels and other foreign material from wheat samples. A mechanical seed counter (Seedburo Equipment Co., Chicago, IL) was used to count ten grams of wheat and the number of kernels in ten grams was converted to 1000-kernel weight. Kernel size distribution was determined using the methodology described by Shuey (1960) in which large kernels remained on sieve No. 7 (2.92 mm; top sieve), medium kernels remained on sieve No. 9 (2.24 mm; middle sieve), and small kernels passed directly through both sieves (bottom).

3.3.3. Dry Milling

Grain samples (150 g) were tempered to 15.5% moisture content 24 h before milling. Samples were milled using a Brabender Quadrumat Jr roller mill (C.W. Brabender Instruments, Inc. South Hackensack, NJ, USA). Three milling fractions were collected: bran, shorts and semolina/farina. Semolina is coarsely ground endosperm from durum and farina is coarsely ground endosperm from hard red spring wheat. Bran, shorts, and semolina/farina were subjected to the traditional Martin wet milling procedure.

3.3.4. Wet Milling

The AACC International Approved Method (38-12.02) for determining wet gluten content in semolina is based on the traditional Martin wet milling method. Therefore, Approved Method 38-12.02 was followed, with slight modifications. Two sets of 10 g samples were washed with ≈300 mL of distilled water to remove starch and soluble material from the developing gluten matrix using a Glutomatic 2200 (Perten Instruments, PerkinElmer Inc., MA, USA). Starch was separated from water soluble material by centrifugation at 2500 x *g* for 15 min. The obtained wet gluten and starch were then dried overnight at 45°C in a convection air oven and weighed. The residual water from both washings was combined and freeze-dried.

3.3.5. DON Analysis

DON content in whole wheat, bran, shorts, semolina/farina, dry gluten, dry starch and freeze-dried-water soluble material was determined by gas chromatography with electron capture detection (GC-ECD) (Appendix Figure B1) as described by Tacke, & Casper (1996) and Simsek, Burgess, Whitney, Gu, & Qian (2012), with a modification of the extraction method using 1 g sample and 8 mL of acetonitrile-water (84:16). The samples were analyzed with an Agilent (Santa Clara, CA, USA) 6890 GC with cool on-

column inlet, DB-5 column and ECD detector. Helium was used as the carrier gas and argon-methane (95:5) was used as the makeup gas. This method had a limit of quantification of 0.2-40 mg/kg.

3.3.6. Statistical Analysis

Durum wheat and hard red spring wheat samples were considered as separate experiments. The experimental design was a randomized complete block with four replications. Each replicate was done on a separate day. Data were subjected to analysis of variance using the 'GLM' procedure in Statistical Analysis System 9.4 (SAS Institute, Cary, NC, USA). Treatment means were separated by Fisher's Protected Least Significant Different (LSD) at P=0.05. Pearson's simple linear correlation coefficient was obtained using the 'CORR' procedure in Statistical Analysis System 9.4 (SAS Institute, Cary, NC, USA).

3.4. Results and Discussion

3.4.1. Proximate Analysis

Mean values for the DON levels in whole wheat flour (WWF) and grain proximate analysis are presented in Table 3.01. HRSW samples 1-3 differed significantly in DON levels, with HRSW 2 having the highest (5.07 mg/kg) and HRSW 3 the lowest (2.76 mg/kg) DON detected levels. HRSW 4 presumably was mycotoxin free since DON was not detected and was included as a control sample. Similarly, DW samples 1-3 differed in DON levels, where DW 1 had the highest DON concentration (10.39 mg/kg). DW 2 (4.88 mg/kg) and DW 3 (3.53 mg/kg) had similar amounts of DON. DON was not detected in DW 4, which was included as a DW control sample.

Samples of HRSW and DW varied in their 1000-kernel weight, kernel protein content, ash content, and falling number (Table 3.01). Variation in these parameters would be expected since samples represent different varieties and growing environments. Part of the environment would be the occurrence of FHB. Samples containing DON were infected with FHB. However, the impact of FHB on grain quality is difficult to ascertain.

The effect of FHB on grain quality is affected by the timing of *Fusarium* infection. Early infection is associated with reduced physical and chemical grain quality. *Fusarium* infections during flowering can affect the kernel development, resulting in small, shriveled and lightweight kernels, leading to an increase in ash content (Matthäus et al., 2011; Schwarz, Schwarz, Zhou, Prom, & Steffenson, 2001). Additionally, a fungus, like *Fusarium*, can be considered an aggressive invader modifying, or even destroying, starch

granules, storage proteins, and cell walls (Matthäus et al., 2011; Wang, Pawelzik, Weinert, & Wolf, 2005). The grain containing DON did not appear to have typical symptoms of FHB infection. DON can be produced from infections late in the kernel development without producing dramatic reductions in grain weight (Del Ponte, Fernandes, & Bergstrom, 2007). In the present study no significant correlations were found between DON levels and 1000-kernel weight, protein content, starch content or ash content for either HRSW or DW samples (Table 3.02).

Interestingly, ash content was greater with HRSW and DW samples that contained DON than control samples or 5-year average. In previous published studies, Matthäus et al. (2011), in agreement with Meyer, Weipert, & Mielke (1986), found ash content increased significantly when grains were infected by *Fusarium*, having a range of ash values of 1.8% to 2.3%, which are greater that those found for HRSW or DW samples used in this research. They attributed increase in ash content by FHB to a reduction in the grain size, resulting in a lower ratio of embryo / husk. The lack of correlation with 1000-kernel weight suggests that ash values probably reflect varietal and environmental differences other than FHB. Although the FHB infection could impact ash content it is likely that the higher ash contents were due to other effects.

Since studies, like the one published by Bechtel, Kaleikau, Gaines, & Seitz (1985), stated that *Fusarium* destroys starch granules leading to a detriment in quality, one might expect that tests like falling number would clearly reflect this damage resulting in low values. All HRSW samples that contained DON had lower falling number values (289-367 sec) than the control (543 sec) or the five-year average (388 sec). Falling number values below 400 sec indicate elevated α-amylase activity. α-Amylase can originate from microorganisms such as *Fusarium* or from the seed (Carson, & Edwards, 2009). α-Amylase in the seed can be due to premature termination of seed development or from onset of germination which under damp conditions can occur before the grain is harvested, otherwise known as preharvest sprouting. The source of α-amylase was not determined. Falling number values were negatively correlated with DON levels (r=- 0.868, *F*=0.001) for HRSW, which indicates a possible relationship with FHB infection. On the other hand, DW samples had falling numbers above 400 sec. Falling number values for DW samples with and without DON (414-474 sec and 468 sec, respectively) were higher than the five year average (380 sec). There was no correlation between DON levels and

falling number regarding DW samples. These results indicated that although DW samples had DON, they did not contain elevated α-amylase activity. These results agree with those found by Wang et al. (2005) and Wang, Pawelzik, Weinert, Zhao, & Wolf (2008). They both reported little or no decrease in falling number when wheat samples were infected by *Fusarium culmorum* during hard dough stages (late infection).

Table 3.01. DON levels detected and proximate analysis (mean ±SD) of HRSW and DW samples.

Sample	DON WWF		1000-kernel weight		Large kernel content		Leco Protein [c]		Total starch		Ash		Falling number	
	----- mg/kg -----		--------- g ---------		---------- % ----------								------- sec ------	
HRSW [a] 1	3.75 b	±0.19	35.1 a	±0.48	81 a	±0.79	15.5 c	±0.15	51.68 a	±1.18	1.66 b	±0.07	367 b	±10.18
HRSW 2	5.07 a	±0.68	30.7 c	±0.00	60 c	±0.87	15.8 b	±0.11	48.84 a	±1.35	1.76 a	±0.04	289 d	±0.00
HRSW 3	2.76 c	±0.33	32.8 b	±0.37	70 b	±0.74	17.6 a	±0.10	49.80 a	±1.20	1.83 a	±0.04	315 c	±21.63
HRSW 4	ND [d]	-	32.4 b	±0.35	44 d	±0.76	14.5 d	±0.16	50.71 a	±1.34	1.52 c	±0.03	543 a	±17.24
5-year Avg.	0.20		31.3		-		14.0		-		1.52		388	
DW [b] 1	10.39 a	±1.63	36.4 c	±0.35	39 c	±0.32	13.8 a	±0.11	54.13 b	±0.62	1.68 a	±0.04	468 a	±27.22
DW 2	4.88 b	±0.87	40.2 a	±0.67	65 a	±0.07	12.1 b	±0.10	55.99 a	±1.33	1.72 a	±0.11	474 a	±15.35
DW 3	3.53 b	±0.28	38.5 b	±1.56	60 b	±1.28	11.9 c	±0.12	56.27 a	±0.88	1.65 a	±0.04	414 b	±8.84
DW 4	ND	-	30.8 d	±0.61	32 d	±0.72	14.0 a	±0.09	52.66 b	±1.48	1.46 b	±0.03	468 a	±8.73
5-year Avg.	1.10		39.6		-		13.6		-		1.59		380	

Means with the same letter within columns for the same wheat type are not significantly different ($P<0.05$).
[a] HRSW: Hard red spring wheat.
[b] DW: Durum wheat.
[c] 12% moisture basis.
[d] ND: Below the limit of quantification (<0.2 mg/kg).

Table 3.02. Correlations between proximate analysis and whole wheat flour (WWF) DON level in HRSW and DW samples.

	1000-kernel weight	Large kernel content	Leco Protein	Total starch	Ash	Falling number
HRSW DON WWF	ns [a]	ns	ns	ns	ns	-0.868***
DW DON WWF	ns	ns	ns	ns	ns	ns

[a] ns: Not significant.
*** Significant at the 0.001 probability level; n=16.

3.4.2. Dry Milling

HRSW and DW samples were milled using a Brabender Quadrumat Jr roller mill that was configured to produce three fractions: farina or semolina fraction from endosperm of HRSW and DW, respectively, shorts, and a bran/germ fraction. Shorts fraction consists of an inseparable mixture of endosperm, bran and germ (Posner, and Hibbs, 2005). HRSW samples varied with their farina and bran extraction but not with the size of their shorts fraction (Table 3.03). With respect to HRSW milling behavior, a positive correlation (r=0.866, *P*=0.001) (Table 3.04) was found between farina extraction and 1000-kernel weight, along with a negative correlation (r=-0.922, *P*=0.001) between bran fraction and 1000-kernel weight. These results agree with Matsuo, & Dexter (1980), who reported a significant correlation between semolina yield and 1000-kernel weight, stating that a larger kernel size will have a greater milling yield. This is also supported by the large kernel content found (Table 3.01), where sample HRSW 4 had the lowest proportion of large kernels among samples. There was a negative correlation between farina extraction and bran/germ fraction (r=-0.984, *P*=0.001). These results indicate that as farina yield increased, shorts and bran yields decreased.

With DW milling, DW samples varied with semolina yield, shorts fraction and bran/germ fraction (Table 3.03). The low semolina yield for control (DW 4) compared to DW 1 – DW 3 probably reflects its low 1000-kernel weight along with the smallest large kernel content (Table 3.01), which resulted in the highest bran/germ fraction (31.1% vs 26.1-27.5%). DW samples had a similar positive (r=0.838, *P*=0.001) and negative (r=- 0.764, *P*=0.001) correlation between 1000-kernel weight trait and semolina yield and 1000-kernel weight and bran fraction, respectively. Similarly, semolina yield was negatively correlated with bran/germ fraction (r=-0.804, *P*=0.001). Although DW samples differed with short fraction, the short fraction did not correlate with semolina yield or with bran fraction. No other analyzed grain factors were correlated with dry milling traits for both HRSW and DW samples.

No correlation was observed between DON levels in HRSW samples and the yield of each dry milled fractions (Table 3.04). Similarly, DON levels in DW samples did not correlate with semolina yield or shorts. Although, DON levels in DW samples was negatively correlated with bran/germ fraction (r=-0.870; *P*=0.001), this correlation was probably an artifact of the low kernel weight of the control sample. Low kernel weight is often associated with small kernel size. Small kernels have high bran-to-endosperm ratio

which often results in low semolina yields and high bran yields. This is an interesting behavior, not observed among the HRSW samples, since it suggests that a high concentration of DON is associated with a brittle bran, which would decrease its yield, while contaminating the other fractions, like semolina, all of which supports the negative correlation between bran fraction and farina/semolina fraction (r=- 0.804, *P*=0.001). Regarding the milling behavior of HRSW and DW samples, it is notable that farina/semolina yields were higher for HRSW when compared to DW samples. This might be attributable to the endosperm hardness, since the harder the endosperm, less farina or semolina is yielded (Endo, Okada, Nagao, & D'Appolonia, 1990).

Table 3.03. Dry milling yields for HRSW and DW samples

Sample	Farina/Semolina fraction	Shorts fraction	Bran fraction
	---------- % ----------		
HRSW 1	63.8 a	9.1 a	27.1 d
HRSW 2	58.6 d	9.2 a	32.2 a
HRSW 3	62.1 b	8.7 a	29.2 c
HRSW 4	60.4 c	8.7 a	30.9 b
HRSW mean	61.2	8.9	29.9
DW 1	57.9 a	16.0 a	26.1 c
DW 2	58.4 a	14.1 b	27.5 b
DW 3	57.9 a	14.6 ab	27.4 b
DW 4	55.1 b	13.8 b	31.1 a
DW mean	57.3	14.6	28.0

Means with the same letter within columns for the same wheat type are not significantly different (*P*<0.05).

Table 3.04. Correlations between WWF DON level and grain factors with extraction of dry milling fractions of HRSW and DW samples.

	Farina/Semolina fraction	Shorts fraction	Bran fraction
HRSW			
DON WWF	ns [a]	ns	ns
1000-kernel weight	0.866***	ns	-0.922***
Leco protein	ns	ns	ns
Total starch	ns	ns	ns
Ash	ns	ns	ns
Falling number	ns	ns	ns
Farina fraction	1	ns	-0.984***
Shorts fraction	ns	1	ns
Bran fraction	-0.984***	ns	1
DW			
DON WWF	ns	ns	-0.870***
1000-kernel weight	0.838***	ns	-0.764***
Leco protein	ns	ns	ns
Total starch	ns	ns	ns
Ash	ns	ns	ns
Falling number	ns	ns	ns
Semolina fraction	1	ns	-0.804***
Shorts fraction	ns	1	ns
Bran fraction	-0.804***	ns	1

[a] ns: Not significant.
*** Significant at the 0.001 probability level; n= 16.

3.4.3. Dry Mill Fractions DON Distribution

The distribution of DON levels for HRSW and DW samples, calculated by using the corresponding dry milled weight and mycotoxin level of each dry milled fraction, is shown in Table 3.05. For HRSW samples, approximately 60% of the DON found in the grain occurred in the bran/germ fraction, 29% in the farina, and 11% in the shorts. For DW samples, approximately 50% of the DON occurred in the bran/germ fraction, 42% in the semolina, and 8% in the shorts. Dry milling DON contaminated wheat has shown that the majority of DON accumulates in the bran outer layer, while a lesser amount of DON was found in the inner endosperm (Cheli et al., 2010; Tibola, Cunha Fernandes, Guarienti, & Nicolau, 2015; Zhang, & Wang, 2014).

In contrast to the distribution of DON in milled fractions where bran > farina/semolina > shorts, the concentration of DON in each fraction was greatest in bran, intermediate with shorts, and least with endosperm. This behavior is explained by the lower amount of shorts obtained in comparison to the farina/semolina, therefore their relative contribution to the overall DON levels is less than the one from the farina/semolina fraction. In other words, DON is more diluted in semolina than in shorts fractions. Considering the concentration within each fraction, all fractions of HRSW and DW samples exceeded the 1 mg/kg limit for food use recommended by the FDA. Shorts and bran are considered coproducts in dry milling and are used in animal feed. Shorts from HRSW1 and HRSW2 and bran from HRSW and DW samples exceeded 5 mg/kg limit for swine and non-ruminant animals. Bran from HRSW1 and DW1 exceeded the 10 mg/kg limit for ruminant animals. Interestingly, the DON content in bran of HRSW 1 was greater than that for HRSW 2, even though grain from HRSW2 had greater concentration than the grain from HRSW 1. This indicates that DON content in the bran and shorts need to be tested regardless of DON content in the grain.

As expected, a positive and highly significant correlation was found between the kernel DON levels and the obtained dry milled fraction DON levels (Table 3.06). A higher concentration of the mycotoxin in the bran is attributed to the *Fusarium* initial colonization in the peripheral parts of the grain, like pericarp and testa, followed by a progressive invasion of the fungi from the surface towards the interior of the kernel (Zhang, & Wang, 2014). The mentioned progressive invasion of the fungi is aided by DON formation, since it triggers H_2O_2 synthesis, a plant defense mechanism, and provokes cell death so

that *Fusarium* can grow in a necrotrophic infection mode (Audenaert et al., 2013). Therefore, DON levels do not necessarily depend on the fungi biomass since DON is a vehicle for invasion, which in case of a late infection can be found in both outer and inner kernel layers (Wegulo, 2012). For late infection, the time for *Fusarium* invasion probably was too short for high levels of hyphae to occur in the endosperm. These findings support previous research about the lower levels of DON in wheat endosperm, while the highest concentration of DON levels was found in wheat bran (Cheli et al., 2010; Thammawong et al., 2011; Tibola et al., 2015; Visconti et al., 2004).

Table 3.05. DON levels detected in WWF, farina/semolina, shorts and bran of HRSW and DW samples.

Sample	DON WWF	DON farina/ semolina	DON shorts	DON bran	DON farina/ semolina [a]	DON shorts [b]	DON bran [c]
	---------- mg/kg ----------				---------- % ----------		
HRSW 1	3.75 b	2.48 a	6.25 a	11.44 a	30	11	59
HRSW 2	5.07 a	2.50 a	6.16 a	9.75 b	28	11	61
HRSW 3	2.76 c	1.66 b	4.79 b	6.72 c	30	12	57
HRSW 4	ND[d]	ND	ND	ND	-	-	-
DW 1	10.39 a	6.46 a	4.80 a	17.96 a	41	8	51
DW 2	4.88 b	3.49 b	2.58 b	8.21 b	44	8	48
DW 3	3.53 b	2.63 c	1.94 b	6.95 c	41	8	51
DW 4	ND	ND	ND	ND	-	-	-

Means with the same letter within columns for the same wheat type are not significantly different (P<0.05).
[a] DON farina/semolina recovery (%)= [Farina/semolina yield (%) x DON level (mg/kg)] / [WWF DON level (mg/kg)] x 100.
[b] DON shorts recovery (%)= [Shorts yield (%) x DON level (mg/kg)] / [WWF DON level (mg/kg)] x 100.
[c] DON bran recovery (%)= [Bran yield (%) x DON level (mg/kg)] / [WWF DON level (mg/kg)] x 100.
[d] ND: Below the limit of quantification (<0.2 mg/kg).

Table 3.06. Correlations between DON level detected in WWF and in dry milling fractions of HRSW and DW samples.

	Farina/ Semolina DON level	Shorts DON level	Bran DON level
HRSW DON WWF	0.940***	0.923***	0.889***
DW DON WWF	0.917***	0.947***	0.981***

*** Significant at the 0.001 probability level; n= 16.

3.4.4. Farina/Semolina Wet Milling

Semolina, the coarsely ground DW endosperm, and farina, the coarsely ground HRSW endosperm, were used in the current study as the starting material for the traditional bench-scale Martin wet milling procedure. The wet milling process resulted in the separation of wheat farina/semolina into starch, gluten, and water solubles.

Starch content and starch extracted (db) in HRSW and DW samples are reported in Table 3.07. Total starch content of DON contaminated farina, HRSW 1 (60.6%) and 2 (61.9%), and the control, HRSW 4 (61.3%), were not significantly different, contrasting with HRSW 3 starch content, which was significantly lower (58.3%) (Table 3.07). On the other hand, the DON contaminated DW samples, DW 2 and 3, had a similar amount of starch, 65.0% and 65.8%, respectively, followed by DW 1 (62.5%), contrasting with the significantly lower 59.6% denoted by the control DW 4. In this regard, Sissons (2008)

reported a mean DW starch content of 67.2%, while Yuan, Chung, Seib, & Wang (1998) reported a mean hard red wheat (HRW) starch content of 65.16%, which are similar to our obtained values.

Extracted starch purity of the did not differ among HRSW samples (78.2 – 81.3%) or DW samples (77.4 – 80.4%), being similar for HRSW and DW. Starch purity of HRSW samples and DW samples are below the commercial starch purity of ≥90% (Ellouzi et al., 2015). However, these differences were expected due to the lack of further washings to the starch fraction. After a single washing, starch slurry from wet milling has been reported to contain fine fiber, soluble protein, and small amounts of fine gluten particles (Langford, & Rist, 1943).

One of the most important criteria for judging wet milling processing is starch yield (Yuan et al., 1998), which in this case was similar among samples denoting no significant differences in either HRSW or DW samples (Table 3.07). The crude starch yield of HRSW samples ranged from 62.4 to 66.8%, while DW samples ranged from 62.4 to 66.4%. The obtained crude starch yields were higher than some reported results, like Yuan et al. (1998) and Wang, & Chung (2001) who obtained 56.1 and 55.8 % starch yield, respectively, from wet milling hard red winter wheat (HRWW). These observed dissimilarities may be due to different wheat classes utilized, in addition to distinct tested wet milling procedures since both Yuan et al. (1998) and Wang, & Chung (2001) started from intact wheat kernels, having a steeping step, followed by further fractionation. On the other hand, Sayaslan, Seib, & Chung (2006) studied wet milling of wheat flour, by hand washing a formed dough, which resulted in similar crude starch yields as ours, of 63.2%. With these results it can be inferred that higher crude starch yields are achieved through wet milling processes starting from wheat flour rather than kernels. On the other hand, the net starch yield was similar among HRSW samples, while DW 3 net starch yield was lower than the rest of the DW samples due to a lower purity obtained.

As seen from Table 3.07, the starch recoveries from HRSW samples, ranging from 81.8 to 88.8%, were statistically similar, whereas the DW samples starch recoveries differed significantly, where DW 3 had the lowest (73.5%) and DW 4 the highest (89.8%) starch recoveries. The obtained starch recoveries broadly agreed with Sayaslan et al. (2006) since their reported starch recoveries ranged 76.0 to 79.9%.

DON was not detected in extracted starch from HRSW or DW (Table 3.07), which is attributed to the high water solubility of DON. These results are in agreement with Lauren, & Ringrose (1997) and Bennett, & Anderson (1978), who found that starch fraction obtained from wet milled mycotoxin contaminated corn was free of measurable quantities of mycotoxins, including DON.

Table 3.07. Content, extraction properties, and DON content of starch obtained by wet milling farina/semolina from HRSW and DW samples.

Sample	Farina / Semolina total starch	Crude starch yield [a]	Extracted starch purity	Net starch yield [b]	Starch recovery [c]	DON level in extracted starch
	%	---------- % ----------				mg/kg
HRSW 1	60.6 a	66.8 a	79.5 a	53.0 a	87.6 a	ND [d]
HRSW 2	61.9 a	62.4 a	81.3 a	50.6 a	81.8 a	ND
HRSW 3	58.3 b	64.3 a	80.4 a	51.7 a	88.8 a	ND
HRSW 4	61.3 a	66.3 a	78.2 a	51.8 a	84.6 a	ND
DW 1	62.5 b	65.9 a	78.8 a	51.9 a	83.1 b	ND
DW 2	65.0 a	65.9 a	79.2 a	52.2 a	80.3 b	ND
DW 3	65.8 a	62.4 a	77.4 a	48.3 b	73.5 c	ND
DW 4	59.6 c	66.4 a	80.4 a	53.4 a	89.8 a	ND

Means with the same letter within columns for the same wheat type are not significantly different (P<0.05).
[a] Crude starch yield (%)= [Extracted starch (g, dm) / Amount of wet milled farina/semolina (g, dm)] x 100.
[b] Net starch yield (%)= {[Extracted starch (g, dm) / Amount of wet milled farina/semolina (g, dm)] x 100} x Starch purity (%).
[c] Starch recovery (%)= [Extracted starch (g, dm) x Starch purity (%)] / [Amount of wet milled farina/semolina (g, dm) x Starch content (%)] x 100.
[d] ND: Below the limit of quantification (<0.2 mg/kg).

Table 3.08. Correlations between starch extraction traits and total starch and DON level in farina/semolina.

	Crude starch yield	Extracted starch purity	Net starch yield	Starch recovery
HRSW – farina				
DON	ns [a]	ns	ns	ns
Total starch	ns	ns	ns	ns
DW – semolina				
DON	ns	ns	ns	ns
Total starch	ns	ns	ns	-0.811***

[a] ns: Not significant.
*** Significant at the 0.001 probability level; n= 16.

Data for protein content and gluten yield and recovery from HRSW and DW samples are shown in Table 3.09. Total protein content (db) was greatest in HRSW 3 (18.8%), intermediate in HRSW 1

(16.6%) and 2 (16.9%), and least in HRSW 4 (15.6%). Farina total protein content was positively and highly correlated with the kernel trait protein content (r=0.961, *P*=0.001). HRSW sample rankings were similar for total protein, crude gluten yield, and net gluten yield. Regarding DW samples, the lowest total protein content corresponded to DW 3 (12.2%) and DW 2 (12.7%), while DW 1 (14.9%) and DW 4 (14.8%) were higher. Similar to farina, semolina protein content was positively correlated with the initial kernel protein content (r=0.949, *P*=0.001). The rankings for DW samples total protein, crude gluten yield, and net gluten yield agree with the total protein content. Crude and net gluten yields appear to be greater for HRSW than DW samples. This is attributed to the higher total protein content in HRSW than DW samples.

Additionally, the purity of the obtained gluten samples was satisfactory as protein content of 75% (% N x 5.7) is the minimum standard for commercial gluten (Sayaslan et al., 2006; Steeneken, & Helmens, 2009). In this regard, both HRSW and DW samples not only met, but exceeded the minimum protein content for being commercialized, the former ranging from 82.2 to 87.7%, and the latter from 83.2 to 84.7%.

One of the key factors for judging a wet milling process, apart from the material purity, is gluten yield (Yuan et al., 1998). Van Der Borght et al. (2005) stated that the typical gluten yields for the Martin process were between 10 and 15%, without specifying the type of wheat utilized. In this case HRSW crude gluten yields were >12.2%, whereas DW crude gluten yields were >9.7%, both falling between the expected yield range. A strong positive correlation between farina/semolina protein content and crude gluten yield explains the significant differences found between samples (Table 3.10), for instance HRSW 3 which had 18.8% db protein also had the highest crude gluten yield (16.5%), likewise DW 1 with 14.9%, db protein in semolina had a high crude gluten yield (11.7%) among DW samples. The same trend was seen for the net gluten yield (Table 3.09), ranging from 10.3 to 14.4% for HRSW samples, and from 8.1 to 9.9% for DW. Additionally, similar gluten yield results are found in literature, like Yuan et al. (1998) who reported 16.1% obtained from HRWW, while Sayaslan et al. (2006) reported 11.9% from a hand-washing dough procedure.

Sayaslan et al. (2006) reported a final protein recovery of 77.9%, which is higher than any of the obtained gluten recoveries of the present study since their gluten fraction was purer (92%) than ours.

However, the purity of our samples can be readily increased by further washings with fresh water, thus increasing the recoveries. Regarding DW samples, the obtained recoveries did not have significant differences among samples, having recoveries from 63.9 to 69.4%. On the other hand, HRSW gluten recoveries had a significant difference, being the sample with the highest farina protein content, HRSW 3, the one with the highest gluten recovery (76.9%), while the rest of the samples denoted a similar gluten recovery.

Gluten extracted from HRSW contained low levels of DON <0.60 mg/kg, while gluten extracted from DW contained no detectable DON. Results found here agree with early work from Lauren, & Ringrose (1997), which by studying the fate of *Fusarium* mycotoxins in corn wet milling found that around 0.3 mg/kg DON and NIV were still detected in gluten fractions. These are promising results since the levels of DON retained in HRSW gluten were below the FDA advisory level for human consumption (1 mg/kg).

From the lack of correlation between the protein content and the DON concentration in farina/semolina, it can be assumed that the protein content was not affected by the presence of DON. These results are in agreement with Wang et al. (2005), who observed that the protein content did not decrease and did not have a significant correlation with different degrees of *Fusarium* infection in wheat. On the other hand, lower concentrations of protein in infected wheat have been reported by Bechtel et al. (1985) and Nightingale, Marchylo, Clear, Dexter, & Preston (1999) which they attributed to the proteases produced by the fungus for a successful colonization of the grain. However, in the case of a late infection, the seed development is not stopped at an early stage, leading to a complete deposition of starch granules and proteins. Little or no visual infection with late infection, which probably reflects limited release of hydrolytic enzymes by the *Fusarium* organism. In this regard, the differences between protein contents among samples are attributable to different cultivars utilized.

Table 3.09. Content, extraction properties, and DON content of gluten obtained by wet milling farina/semolina from HRSW and DW samples.

Sample	Farina / Semolina total protein	Crude gluten yield [a]	Extracted gluten purity	Net gluten yield [b]	Gluten recovery [c]	Extracted gluten DON level
	%	---------- % ----------				mg/kg
HRSW 1	16.6 b	14.0 b	83.4 b	11.6 b	70.3 b	0.60
HRSW 2	16.9 b	14.2 b	82.2 c	11.7 b	69.0 b	0.35
HRSW 3	18.8 a	16.5 a	87.7 a	14.4 a	76.9 a	0.34
HRSW 4	15.6 c	12.3 c	83.6 b	10.3 c	66.0 b	ND [d]
DW 1	14.9 a	11.7 a	84.7 a	9.9 a	66.3 a	ND
DW 2	12.7 b	9.7 b	83.2 b	8.1 b	63.9 a	ND
DW 3	12.2 b	10.2 b	83.2 b	8.5 b	69.4 a	ND
DW 4	14.8 a	11.7 a	84.6 a	9.9 a	66.8 a	ND

Means with the same letter within columns for the same wheat type are not significantly different (P<0.05).
[a] Crude gluten yield (%)= [Extracted gluten (g, dm) / Amount of wet milled farina/semolina (g, dm)] x 100.
[b] Net gluten yield (%)= {[Extracted gluten (g, dm) / Amount of wet milled farina/semolina (g, dm)] x 100} x Gluten purity (%).
[c] Gluten recovery (%)= [Extracted gluten (g, dm) x Gluten purity (%)] / [Amount of wet milled farina/semolina (g, dm) x Protein content (%)] x 100.
[d] ND: Below the limit of quantification (<0.2 mg/kg).

Table 3.10. Correlations between gluten extraction traits and total protein and DON level in farina/semolina of HRSW and DW samples.

	Crude gluten yield	Extracted gluten purity	Net gluten yield	Gluten recovery
HRSW – farina				
DON	ns [a]	ns	ns	ns
Total protein	0.924***	0.763***	0.904***	ns
DW – semolina				
DON	ns	ns	ns	ns
Total protein	0.816***	0.909***	0.791***	ns

[a] ns: Not significant.
*** Significant at the 0.001 probability level; n= 16.

Water soluble material was quantified by freeze-drying the wash water. Regarding HRSW, the amount of obtained freeze-dried water solubles ranged from 0.4 to 0.5 g/10 g or an average of 4.7% of farina and was similar among HRSW samples (Table 3.11). The water soluble content in DW samples was 0.7 g/10 g for DW 1 and 2, followed by 0.6 g for DW 4, and 0.5 g for DW 3 for an average of 5.8% of semolina. The water soluble fraction yields obtained for HRSW (4.1-5.1%) and for DW (5.0-6.4%) fall

within the estimated ranges for wheat flours of 5% to 15% obtained by Hamer, Weegels, Marseille, & Kelfkens, (1989) and Sayaslan et al. (2006).

DON levels were very high in the freeze-dried water soluble fractions from farina and semolina (32.9-49.3 mg/kg and 46.2-99.3 mg/kg, respectively). For both wheat species, neither the amount of water solubles nor their yield were correlated with the DON levels in the farina/semolina (Table 3.12). As expected, DON levels in the water soluble fraction and the initial DON levels in HRSW farina ($r=0.942$, $P=0.001$) and DW semolina ($r=0.982$, $P=0.001$) were significantly and positively correlated (Table 3.12).

For both wheat species, HRSW and DW, 88 to 99% of the recovered DON was found in the water soluble fraction (Table 3.11), which again confirms how an advantage can be taken from DON's high water solubility in order to obtain mycotoxin free wheat co-products. From studies done on wet milled mycotoxin contaminated corn, it has been found the water soluble DON and NIV are mainly found in the liquid fraction, whereas ZEN and aflatoxins, which have low water solubility, tend to be concentrated in fiber and gluten (Bennett, & Anderson, 1978; Bennett, Richard, & Eckhoff, 1996; Lauren, & Ringrose, 1997).

Table 3.11. Content, extraction properties, and DON content of water solubles obtained by wet milling farina/semolina from HRSW and DW samples.

	Freeze-dried water soluble fraction	Crude water soluble fraction yield [a]	DON level in water soluble fraction	DON recovery from water soluble fraction [b]
Sample	g/10 g	%	mg/kg	%
HRSW 1	0.5 a	5.1 a	47.9 a	98.9
HRSW 2	0.5 a	4.6 a	49.3 a	91.1
HRSW 3	0.5 a	5.0 a	32.9 b	99.6
HRSW 4	0.4 a	4.1 a	ND [c]	-
DW 1	0.7 a	6.4 a	99.3 a	98.4
DW 2	0.7 a	6.2 a	55.6 b	99.5
DW 3	0.5 c	5.0 c	46.2 c	87.7
DW 4	0.6 b	5.6 b	ND	-

Means with the same letter within columns for the same wheat type are not significantly different (P<0.05).
[a] Crude water soluble fraction yield (%)= [Extracted water solubles (g, dm) / Amount of wet milled farina/semolina (g, dm)] x 100.
[b] Water solubles DON recovery (%)= [Water solubles yield (%) x DON level (mg/kg)] / [Farina/semolina DON level (mg/kg)] x 100.
[c] ND: Below the limit of quantification (<0.2 mg/kg).

Table 3.12. Correlations between water soluble fraction traits and DON level in farina/semolina in HRSW and DW samples.

	Freeze-dried water soluble fraction	Water soluble fraction yield	DON level, water soluble fraction
HRSW DON farina	ns [a]	ns	0.942***
DW DON semolina	ns	ns	0.982***

[a] ns: Not significant.
*** Significant at the 0.001 probability level; n= 16.

3.4.5. Shorts and Bran Wet Milling

The milling co-products comprise of bran (outermost layers of the kernel and aleurone layer) and shorts (mixture of fine particles of bran, germ, and unseparated flour) (Kim, Flores, Chung, & Bechtel, 2003; Sarfaraz, Azizi, Gavlighi, & Barzegar, 2017). The co-products have been intended mainly for animal feed and in small proportion for human food, but they have been traditionally considered as agricultural waste (Apprich et al., 2014; Sarfaraz et al., 2017). In this regard, co-products were not subjected to a proximate analysis, it was rather intended to test if by wet milling the co-products the fate of DON was the same as for the flour, so as to consider a potential use of the co-products rather than just dispose them.

After wet milling the shorts, HRSW samples did not have significant differences in the amount of extracted starch (Table 3.13), which represented a crude yield that ranged from 54.4 to 63.7%. Regarding DW samples, the amount of extracted starch was similar for DW 2 (64.0%), 3 (64.9%), and 4 (63.2%), which represented ≈64% crude starch yield, whereas DW 1 extracted starch accounted for 58.8% of shorts yielded material. The obtained crude yields for starch were roughly similar to those seen when wet milling farina/semolina. These findings are reasonable since shorts are the wheat dry milling co-products that account for the highest starch content due to the amount of reduced endosperm particles that constitutes them (Kim et al., 2003; Sarfaraz et al., 2017).

The amount of gluten was lower and water soluble material was higher when compared to farina/semolina (Tables 3.09 and 3.13). These results probably reflect the presence of more fiber (bran) particles that were not removed from endosperm during dry milling. Fiber would interrupt the proper development of a gluten network (Khalid, Ohm, & Simsek, 2017). Crude yield for gluten extracted from shorts fraction of HRSW samples ranged from 9.5 to 12.8%, while that from DW samples ranged from 7.6 to 10.8%. The differences in yield are attributed to the different cultivars studied. With respect to the water soluble material, HRSW samples had similar crude yields 7.2 to 8.0%, while DW samples varied from 8.1 to 9.6%. None of the extractions and calculated crude yields of starch, gluten and water solubles obtained from the shorts wet milling were significantly correlated with the initial DON level in shorts (Table 3.14).

DON was not detected in any fraction except for the freeze dried water soluble material. The initial level of DON in shorts was significantly and positively correlated to DON level found in freeze-dried water soluble material for both HRSW (r=0.991, *P*=0.001) and DW (r=0.895, *P*=0.001) samples (Table 3.14). Average recoveries greater than 90 % were obtained from the freeze-dried water soluble material, which explains why the levels of DON in the shorts starch and gluten fractions were below the limit of quantification.

Table 3.13. Content, extraction properties, and DON content in starch, gluten and freeze-dried water solubles obtained by wet milling shorts from HRSW and DW samples.

Sample	Extracted starch in shorts fraction	Extracted gluten in shorts fraction	Freeze-dried water soluble in shorts fraction	Crude shorts starch yield [a]	Crude shorts gluten yield [b]	Crude shorts water soluble fraction yield [c]	DON level in shorts water soluble fraction	DON recovery from shorts water soluble fraction [d]
	---------- g / 10 g ----------			---------- % ----------			mg/kg	%
HRSW 1	3.0 a	0.6 a	0.4 a	60.0 a	12.0 a	8.0 a	77.0 a	98.2
HRSW 2	3.1 a	0.5 b	0.4 a	60.9 a	10.2 b	7.6 a	74.5 a	92.2
HRSW 3	2.8 a	0.7 a	0.4 a	54.4 a	12.8 a	7.2 a	53.9 b	81.4
HRSW 4	3.2 a	0.5 b	0.4 a	63.7 a	9.5 b	7.3 a	ND [e]	-
DW 1	2.9 b	0.6 a	0.5 a	58.8 b	10.8 a	9.6 a	44.4 a	88.8
DW 2	3.2 a	0.4 c	0.5 a	64.0 a	7.6 c	9.5 a	23.4 b	86.2
DW 3	3.3 a	0.5 b	0.4 b	64.9 a	9.2 b	8.1 b	25.1 b	104.5
DW 4	3.2 a	0.6 a	0.5 a	63.2 a	10.4 a	8.9 a	ND	-

Means with the same letter within columns for the same wheat type are not significantly different (P<0.05).
[a] Crude shorts starch yield (%)= [Extracted starch (g, dm) / Amount of wet milled shorts (g, dm)] x 100.
[b] Crude shorts gluten yield (%)= [Extracted gluten (g, dm) / Amount of wet milled shorts (g, dm)] x 100.
[c] Crude shorts water soluble fraction yield (%)= [Extracted water sol. (g, dm) / Amount of wet milled shorts (g, dm)] x 100.
[d] Water soluble fraction DON recovery (%)= [Water solubles yield (%) x DON level (mg/kg)] / [Shorts DON level (mg/kg)] x 100.
[e] ND: Below the limit of quantification (<0.2 mg/kg).

Table 3.14. Correlations between starch, gluten and water solubles extraction traits and DON level in shorts from HRSW and DW samples.

	Extracted starch in shorts fraction	Extracted gluten in shorts fraction	Freeze-dried shorts water soluble fraction	Crude shorts starch yield	Crude shorts gluten yield	Crude shorts water soluble fraction yield	DON level, shorts water soluble fraction l
HRSW DON shorts	ns [a]	ns	ns	ns	ns	ns	0.991***
DW DON shorts	ns	ns	ns	ns	ns	ns	0.895***

[a] ns: Not significant.
*** Significant at the 0.001 probability level.

After wet milling the bran, three fractions were collected: starch, water solubles and de-starched bran. It was noteworthy that of the three dry milled fractions that underwent wet milling, bran was the only one that did not yield gluten, but this is explained since bran proteins primarily consist of albumins and globulins, rather than glutenins and gliadins as in the endosperm (Apprich et al., 2014). Therefore, in order to obtain the separation of proteins from bran, either acidic or alkaline conditions would need to be met (Roberts, Simmonds, Wootton, & Wrigley, 1985). HRSW samples yielded around 30.6%, 55.7% and 13.5% of starch, de-starched bran and water soluble material, respectively, without significant differences among samples (Table 3.15). On the other hand, DW samples yielded around 45.7%, 37.7% and 16.6% of starch, de-starched bran and water soluble material, respectively, without having significant differences only for starch (Table 3.15).

With the exception of HRSW 4 and DW 4, DON was detected in all bran wet milling fractions. Wet milling bran greatly reduced DON levels (Tables 3.05 and 3.15). For HRSW samples, DON ranged from 0.78-1.09 mg/kg in extracted starch and 0.58-1.59 mg/kg in de-starched bran. Similarly, for DW samples DON ranged from 0.73-2.08 mg/kg in extracted starch and 0.07-0.79 mg/kg in de-starched bran. These data indicate that removing DON from bran fraction is more difficult than removing DON from farina/semolina or from shorts fractions. This difficulty probably relates to the lack of dough forming proteins in the bran. Dough formation is a key characteristic of the Martin process. These results might also reflect the water holding capacity of bran. Bran might be holding water that contained DON due to the hydroxyl groups in celluloses and pentosans (Khalid, Ohm, & Simsek, 2017; Majzoobi, Pashangeh, Aminlari, & Farahnaky, 2014; Sarfaraz et al., 2017). It is anticipated that DON could be removed if more water was used during the washing of the bran.

Regarding the fate of DON, the three wet milled fractions from bran had detectable levels of DON (Table 3.15), and most of the DON in the extracted fractions was highly correlated with the initial DON levels in bran (Table 3.16). This may also be related to the fact that each fraction was obtained without further purification, so as to ensure that for instance the starch fraction was not highly contaminated with bran or other components that might carry the mycotoxin. As expected, most of the mycotoxin levels were found in the freeze-dried water soluble fraction for both wheat species.

Table 3.15. Content, extraction properties, and DON content in extracted starch, de-starched bran, and freeze dried water solubles from bran obtained by wet milling bran fraction from HRSW and DW samples.

Sample	Extracted starch in bran fraction	Extracted de-starched bran in bran fraction	Freeze-dried water soluble in bran fraction	Crude bran starch yield [a]	Crude de-starched bran yield [b]	Crude bran water soluble fraction yield [c]	DON level in bran starch	DON level in de-starched bran	DON level in bran water soluble fraction	DON recovery from bran starch [d]	DON recovery from de-starched bran [e]	DON recovery from bran water soluble fraction [f]
	---------- g / 10 g ----------			---------- % ----------			---------- mg/kg ----------			---------- % ----------		
HRSW 1	1.5 a	1.8 a	0.7 a	29.0 a	56.9 a	13.5 a	1.09 a	1.59 a	66.98 a	2.8	7.9	79.0
HRSW 2	1.6 a	1.9 a	0.7 a	30.7 a	57.1 a	12.8 a	0.87 b	1.24 a	58.46 b	2.7	7.2	76.9
HRSW 3	1.5 a	1.9 a	0.7 a	29.4 a	56.7 a	13.6 a	0.78 b	0.58 b	45.97 c	3.4	4.8	93.0
HRSW 4	1.7 a	1.6 a	0.7 a	33.4 a	51.9 a	13.9 a	ND [g]	ND	ND	-	-	-
DW 1	2.3 a	0.8 c	0.9 a	45.6 a	35.4 c	18.5 a	2.08 a	0.79 a	76.20 a	5.3	1.6	78.3
DW 2	2.3 a	0.8 c	0.9 a	46.2 a	35.8 c	17.3 a	0.83 b	0.23 b	41.60 b	4.7	1.0	87.9
DW 3	2.3 a	0.9 b	0.9 a	45.7 a	37.5 b	16.6 a	0.73 b	0.07 b	41.38 b	4.8	0.4	98.9
DW 4	2.3 a	1.1 a	0.7 b	45.4 a	42.0 a	13.8 b	ND	ND	ND	-	-	-

Means with the same letter within columns for the same wheat type are not significantly different ($P<0.05$).

[a] Crude bran starch yield (%)= [Extracted starch (g, dm) / Amount of wet milled shorts (g, dm)] x 100.

[b] Crude de-starched bran yield (%)= [Extracted gluten (g, dm) / Amount of wet milled shorts (g, dm)] x 100.

[c] Crude bran water soluble fraction yield (%)= [Extracted water sol. (g, dm) / Amount of wet milled shorts (g, dm)] x 100.

[d] Starch DON recovery (%)= [Starch yield (%) x DON level (mg/kg)] / [Bran DON level (mg/kg)] x 100.

[e] De-starched bran DON recovery (%)= [Washed bran yield (%) x DON level (mg/kg)] / [Bran DON level (mg/kg)] x 100.

[f] Water soluble fraction DON recovery (%)= [Water solubles yield (%) x DON level (mg/kg)] / [Bran DON level (mg/kg)] x 100.

[g] ND: Below the limit of quantification (<0.2 mg/kg).

Table 3.16. Correlations between extracted bran starch, de-starched bran, and freeze dried water solubles and DON level in bran from HRSW and DW samples.

	Extracted starch in bran fraction	Extracted de-starched bran in bran fraction	Freeze-dried water soluble fraction	Crude bran starch yield	Crude de-starched bran yield	Crude bran water soluble fraction yield	DON level, bran starch	DON level, de-starched bran	DON level, bran water soluble fraction
HRSW DON bran	ns [a]	ns	ns	ns	ns	ns	0.871***	ns	0.969***
DW DON bran	ns	-0.804***	0.814***	ns	-0.800***	0.821***	0.994***	0.882***	0.974***

[a] ns: Not significant.
*** Significant at the 0.001 probability level; n= 16.

3.5. Conclusions

In general, after dry milling the three collected fractions, farina/semolina, shorts and bran had DON contamination higher than 1 mg/kg. Wet milling was effective in removing or decreasing DON levels from all three dry milled fractions. From wet milling farina/semolina, an average of 97% and 95% of DON was recovered in the freeze-dried water solubles fraction from HRSW and DW samples, respectively. Therefore, no DON levels were detected in the starch obtained from farina/semolina wet milling, while gluten extracted from farina contained low levels of DON (<0.60 mg/kg) and gluten extracted from semolina had no detectable DON. Similar results were found for the shorts fraction, where an average of 91% and 93% of DON was recovered in the freeze-dried water soluble fraction for HRSW and DW, respectively. After bran underwent wet milling, the distribution of DON concentration found in the starch, de-starched bran, and water soluble fraction was 3%, 7% and 83% for HRSW samples, and 5%, 1%, and 88% for DW samples, respectively.

3.6. References

AACC International. *Approved methods of analysis* (11th Ed.). Methods, 08-01.01, 44-15.02, 46-30.01, 56-81.03, and 76-13.01. Available online only. St. Paul, MN: AACCI.

Anonymous. (2017). U.S. Hard red spring wheat Regional Quality Report 16. pp. 36.

Anonymous. (2017). U.S. Durum wheat Regional Quality Report 16. pp. 24.

Apprich, S., Tirpanalan, O., Hell, J., Reisinger, M., Bohmdorfer, S., Siebenhandl-Ehn, S., Novalin, S., & Kneifel, W. (2014). Wheat bran-based biorefinery 2: Valorization of products. *Food Science and Technology*, *56*, 222-231.

Audenaert, K., Vanheule, A., Hofte, M., & Haesaert, G. (2013). Deoxynivalenol: A major player in the multifaceted response of Fusarium to its environment. *Toxins*, *6*, 1-19.

Bechtel, D.B., Kaleikau, L. A., Gaines, R. L., & Seitz, L. M. (1985). The effects of Fusarium graminearum infection on wheat kernels. *Cereal Chemistry*, *62*, 191-197.

Bennett, G. A., & Anderson, R. A. (1978). Distribution of aflatoxin and/or zearalenone in wet-milled corn products: A review. *Journal of agricultural and food chemistry*, *26*, 1055-1060.

Bennett, G. A., Richard, J. L., & Eckhoff, S. R. (1996). Distribution of fumonisins in food and feed products prepared from contaminated corn. In L. S. Jackson, J. W. DeVries, and L. B. Bullerman (Eds.), *Fumonisins in food* (pp. 317-322). New York: Plenum Press.

Carson, G. R., & Edwards, N. M. (2009). Criteria of wheat and flour quality. In K. Khan, and P. R. Shewry (Eds.), *Wheat chemistry and technology* (pp. 97-118). St Paul, MN: AACCI.

Champeil, A., Doré, T., & Fourbet, J. F. (2004). Fusarium head blight: epidemiological origin of the effects of cultural practices on head blight attacks and the production of mycotoxins by Fusarium in wheat grains. *Plant Science*, *166*, 1389-1415.

Cheli, F., Campagnoli, A., Ventura, V., Brera, C., Berdini, C., Palmaccio, E., & Dell'Orto, V. (2010). Effects of industrial processing on the distributions of deoxynivalenol, cadmium and lead in durum wheat milling fractions. *Food Science and Technology*, *43*, 1050-1057.

Cheli, F., Pinotti, L., Rossi, L., & Dell'Orto, V. (2013). Effect of milling procedures on mycotoxin distribution in wheat fractions: A review. *Journal of Food Science and Technology*, *54*, 307-314.

De Angelis, E., Monaci, L., Pascale, M., & Visconti, A. (2013). Fate of deoxynivalenol, T-2, and HT-2 toxins and their glucoside conjugates from flour to bread: an investigation by high-performance liquid chromatography high-resolution mass spectrometry. *Food Additives & Contaminants*, *30*, 345-355.

Del Ponte, E. M., Fernandes, J. M., & Bergstrom, G. C. (2007). Influence of growth stage on Fusarium Head Blight and deoxynivalenol production in wheat. *Journal of Phytopathology*, *155*, 577-581.

Dexter, J. E., Marchylo, B. A., Clear, R. M., & Clarke, J. M. (1997). Effect of Fusarium Head Blight on semolina milling and pasta-making quality of durum wheat. *Cereal Chemistry*, *74*, 519-525.

Döll, S., & Dänicke, S. (2011). The Fusarium toxins deoxynivalenol (DON) and zearalenone (ZON) in animal feeding. *Preventive Veterinary Medicine*, *102*, 132-145.

Ellouzi, S. Z., Driss, D., Maktouf, S., Neifar, M., Kobbi, A., Kamoun, H., Chaabouni, S. E., & Ghorbel, R. E. (2015). Isolation and characterization of starch from industrial fresh pasta by-product and its potential use in sugar-snap cookie making. *Journal Food Science Technology, 52*, 5754-5762.

Endo, S., Okada, K., Nagao, S., & D'Appolonia, B. L. (1990). Quality characteristics of hard red spring and winter wheats I. Differentiatoin by reversed-phase high-performance liquid chromatography and milling properties. *Cereal Chemistry*, *67*, 480-485.

Gärtner, B. H., Munich, M., Kleijer, G., & Mascher, F. (2008). Characterization of kernel resistance against Fusarium infection in spring wheat by baking quality and mycotoxin assessments. *European Journal of Plant Pathology*, *120*, 61-68.

Hammer, R. J., Weegels, P. L., Marseille, J. P., & Kelfkens, M. (1989). A study of the factors affecting the separation of wheta flour into starch and gluten. In Y. Pomeranz (Ed.), *Wheat is unique* (pp. 467-477). St Paul, MN: AACCI.

Hart, L. P., & Braselton, W. E. Jr. (1983). Distribution of vomitoxin in dry milled fractions of wheat infected with Gibberella zeae. *Journal of Agricultural and Food Chemistry*, *31*, 657-659.

Kang, Z., & Buchenauer, H. (2002). Studies on the infection process of Fusarium culmorum in wheat spikes: Degradation of host cell wall components and localization of trichothecene toxins in infected tissue. *European Journal of Plant Pathology*, *108*, 653-660.

Kaushik, G. (2015). Effect of processing on mycotoxin content in grains. *Critical Reviews in Food Science and Nutrition*, *55*, 1672-1683.

Khalid, K. H., Ohm, J.-B., & Simsek, S. (2017). Whole wheat bread: Effect of bran fractions on dough and end-product quality. *Journal of Cereal Science*, *78*, 48-56.

Khaneghah, A. M., Martins, L. M., von Hertwig, A. M., & Bertolo, R. (2018). Deoxynivalenol and its masked forms: Characteristics, incidence, control and fate during wheat and wheat based products processing – A review. *Trends in Food Science & Technology*, *71*, 13-24.

Kim, Y. S., Flores, R. A., Chung, O. K., & Bechtel, D. B. (2003). Physical, chemical, and thermal characterization of wheat flour milling coproducts. *Journal of Food Process Engineering*, *26*, 469-488.

Kushiro, M. (2008). Effects of milling and cooking processes on the deoxynivalenol content in wheat. *International Journal of Molecular Sciences*, *9*, 2127-2145.

Lancova, K., Hajslova, J., Kostelanska, M., Kohoutkova, J., Nedelnik, J., Moravcova, H., & Vanova, M. (2008). Fate of trichothecenes mycotoxins during the processing: milling and baking. *Food Additives and Contaminants*, *25*, 650-659.

Langford, C. T., & Rist, C. E. (1943). Starch from wheat. *Yearbook Separate*, *1976*, 744-749.

Lauren, D. R., & Ringrose, M. A. (1997). Determination of the fate of three Fusarium mycotoxin through wet-milling of maize using an improved HPLC analytical technique. *Food Additives & Contaminants*, *14*, 435-443.

Majzoobi, M., Pashangeh, S., Aminlari, L., & Farahnaky, A. (2014). Modelling the effects of the quantity and particle size of wheat bran on some properties of bread dough using response surface methodology. *International Journal of Food Engineering*, *10*, 511-519.

Maningat, C. C., Seib, P. A., Bassi, S. D., Woo, K. S., & Lasater, G. D. (2009). Wheat starch: Production, properties, modification and uses. In J. BeMiller, and R. Whistler (Eds.), *Starch chemistry and technology* (pp. 441–510). USA: Elsevier Inc.

Matsuo, R. R., & Dexter, J. E. (1980). Relationship between some durum wheat physical characteristics and semolina milling properties. *Canadian Journal of Plant Science, 60*, 49-53.

Matthäus, K., Dänicke, S., Vahjen, W., Simon, O., Wang, J., Valenta, H., Meyer, K., Strumpf, A., Ziesenib, H., & Flachowsky, G. (2011). Progression of mycotoxin and nutrient concentrations in wheat after inoculation with Fusarium culmorum. *Archives of Animal Nutrition*, *58*, 19-35.

Meyer, D., Weipert, D., & Mielke, H. (1986). Influencing the quality of wheat by infecting with Fusarium culmorum. *Getreide, Mehl und Brot*, *40*, 35-39.

Nightingale, M. J., Marchylo, B. A., Clear, R. M., Dexter, J. E., & Preston, K. R. (1999). Fusarium head blight: Effect of fungal proteases on wheat storage proteins. *Cereal Chemistry*, *76*, 150-158.

Petska, J. J. (2007). Deoxynivalenol: Toxicity, mechanisms and animal health risks. *Animal Feed Science and Technology*, *137*, 283-298.

Posner, E. S., & Hibbs, A. N. (2005). *Wheat flour milling* (pp. 489). St Paul, MN: AACCI.

Roberts, P. J., Simmonds, D. H., Wootton, M., & Wrigley, C. W. (1985). Extraction of protein and solids from wheat bran. *Journal of the Science of Food and Agriculture*, *36*, 5-10.

Sarfaraz, A., Azizi, M. H., Gavlighi, H. A., & Barsegar, M. (2017). Physicochemical and fuctional characterization of wheat milling co-products: Fine grinding to achive high fiber antioxidant-rich fractions. *Journal of Cereal Science*, *77*, 228-234.

Sayaslan, A. (2004). Wet-milling of wheat flour: Industrial processes and small-scale test methods. *Journal of Food Science and Technology*, *37*, 499-515.

Sayaslan, A., Seib, P. A., & Chung, O. K. (2006). Wet-milling properties of waxy wheat flours by two laboratory methods. *Journal of Food Engineering*, *72*, 167-178.

Schwarz, P. B., Schwarz, J. G., Zhou, A., Prom, L. K., & Steffenson, B. J. (2001). Effect of Fusarium graminearum and Fusarium poae infection on barley and malt quality. *Monatsschrift fur Brauwissenschaft*, *3*, 55-63.

Scott, P. M., Kanhere, S. R., Lau, P. Y., Dexter, J. E., & Greenhalgh, R. (1983). Effects of experimental flour milling and breadmaking on retention of deoxynivalenol (vomitoxin) in hard red spring wheat. *Cereal Chemistry*, *60*, 421-424.

Seitz, L. M., & Bechtel, D. B. (1985). Chemical, physical and microscopical studies of scab-infected hard red winter wheat. *Journal of Agricultural and Food Chemistry*, *33*, 373-379.

Seitz, L. M., Yamazaki, W. T., Clements, R. L., Mohr, H. E., & Andrews, L. (1985). Distribution of deoxynivalenol in soft wheat mill streams. *Cereal Chemistry*, *62*, 467-469.

Shuey, W. C. (1960). A wheat sizing technique for predicting flour milling yield. *Cereal Science Today*, *5*, 71-75.

Simsek, S., Burgess, K., Whitney, K. L., Gu, Y., & Qian, S. Y. (2012). Analysis of deoxynivalenol and deoxynivalenol-3-glucoside in wheat. *Food Control*, *26*, 287-292.

Sissons, M. (2008). Role of durum wheat composition on the quality of pasta and bread. *Food*, *2*, 75-90.

Sobrova, P., Adam, V., Vasatkova, A., Beklova, M., Zeman, L., & Kizek, R. (2010). Deoxynivalenol and its toxicity. *Interdisciplinary Toxicology*, *3*, 94-99.

Steeneken, P. A. M., & Helmens, H. J. (2009). Laboratory-scale dry/wet-milling process for the extraction of starch and gluten from wheat. *Starch-Stärke*, *61*, 389-397.

Tacke, B. K., & Casper, H. H. (1996). Determination of deoxynivalenol in wheat, barley, and malt by column cleanup and gas chromatography with electron capture detection. *Journal of AOAC International*, *79*, 472-475.

Thammawong, M., Okadome, H., Shiina, T., Nakagawa, H., Nagashima, H., Nakajima, T., & Kushiro, M. (2011). Distinct distribution of deoxynivalenol, nivalenol, and ergosterol in Fusarium-infected Japanese soft red winter wheat milling fractions. *Mycopathologia*, *172*, 323-330.

Tibola, C. S., Cunha Fernandes, J. M., Guarienti, E. M., & Nicolau, M. (2015). Distribution of Fusarium mycotoxins in wheat milling process. *Food Control*, *53*, 91-95.

Van Der Borght, A., Goesaert, H., Veraverbeke, W. S., & Delcour, J. A. (2005). Fractionation of wheat and wheat flour into starch and gluten: overview of the main processes and the factors involved. *Journal of Cereal Science*, *41*, 221-237.

Vidal, A., Morales, H., Sanchis, V., Ramos, A. J., & Marín, S. (2014). Stability of DON and OTA during the breadmaking process and determination of process and performance criteria. *Food Control*, *40*, 234-242.

Visconti, A., Haidukowski, E. M., Pascale, M., & Silvestri, M. (2004). Reduction of deoxynivalenol during durum wheat processing and spaghetti cooking. *Toxicology Letters*, *153*, 181-189.

Wang, D., & Chung, D. S. (2002). Study of a small-scale laboratory wet-milling procedure for wheat. *Cereal Chemistry*, *79*, 115-119.

Wang, J., Pawelzik, E., Weinert, J., & Wolf, G. A. (2005). Impact of Fusarium culmorum on the polysaccharides of wheat flour. *Journal of Agricultural and Food Chemistry*, *53*, 5818-5823.

Wang, J., Pawelzik, E., Weinert, J., Zhao, Q., & Wolf, G. A. (2008). Factors influencing falling number in winter wheat. *European Food Research and Technology*, *226*, 1365-1371.

Wang, J., Wieser, H., Pawelzik, E., Weinert, J., Keutgen, A. J., & Wolf, G. A. (2005). Impact of the fungal protease produced by Fusarium culmorum on the protein quality and breadmaking properties of winter wheat. *European Food Research and Technology*, *220*, 552-559.

Wegulo, S. N. (2012). Factors influencing Deoxynivalenol accumulation in small grain cereals. *Toxins*, *4*, 1157-1180.

Wilson, W. W., McKee, G., Nganje, W., Dahl, B., and Bangsund, D. (2017). Economic impact of USWBSI's scab initiative to reduce FHB. Agribusiness & Applied Economics 774. Available online http://ageconsearch.umn.edu/record/264672. North Dakota State University, Fargo.

Wu, Q., Kuca, K., Humpf, H., Klimova, B., & Cramer, B. (2017). Fate of deoxynivalenol and deoxynivalenol-3-glucoside during cereal-based thermal food processing: A review study. *Mycotoxin Research*, *33*, 79-91.

Yuan, J., Chung, D. S., Seib, P. A., & Wang, Y. (1998). Effect of steeping conditions on wet-milling characteristics of hard red winter wheat. *Cereal Chemistry*, *75*, 145-148.

Zhang, H., & Wang, B. (2014). Fate of deoxynivalenol and deoxynivalenol-3-glucoside during wheat milling and Chinese steamed bread processing. *Food Control*, *44*, 86-91.

4. WET MILLING PROCESS AFFECTS DEOXYNIVALENOL REMOVAL FROM FARINA AND SEMOLINA

4.1. Abstract

Wheat wet milling typically begins with forming a dough from flour, after which the dough is kneaded in excess water resulting in starch, gluten, and water soluble fractions. Deoxynivalenol (DON), a *Fusarium* mycotoxin, is highly water soluble and its presence reduces the value of the wheat or wheat products. The aim of this research was to compare the effectiveness of three laboratory scale wet milling processes (Martin, medium shear, and high shear) in removing DON from wheat. The three wet milling processes were effective in the removal of DON from contaminated wheat. Regardless the wet milling process tested, the extracted starch and gluten did not have detectable DON levels. On average, water soluble fraction of HRSW and DW contained 90 and 47% of DON. Significant (P<0.05) differences were detected from the starch and gluten net yields and recoveries of the three studied wet milling processes. This study indicates the three wet milling processes did not differ in their effectiveness in reducing or eliminating DON from wheat starch and gluten; thus, increasing the value of a low value raw material.

4.2. Introduction

Wheat (*Triticum aestivum* L.), with a worldwide production of 749 million tonnes in 2016 (FAO, 2018), is one of the main cultivated crops. Wheat-based products are one of the primary sources of plant proteins and calories in the human diet (Jones, 2005). Wheat kernels are generally dry milled into wheat flour for further processing into foodstuffs, such as pasta and diverse types of bread (Khaneghah, Martins, von Hertwig, Bertoldo, & Sant'Anna, 2018). Industrial wheat applications, which account for approximately 6% of all wheat utilizations, encompass the use of starch and gluten which is obtained by wet milling.

The wheat wet-milling process separates starch from gluten, and begins after dry milling the wheat grain to separate endosperm from bran and germ. Thanks to the emergence and prevalence of value added ingredients in products, like clean labeled products, wheat starch and gluten are being sold as a commodity with a wide range of uses around the world (Day, Augustin, Batey, & Wrigley, 2006). In this regard, starch is mainly used as starch derived sweeteners, native starch, and modified starches such as cross-linked, substituted, or oxidized starch (Waterschoot, Gomand, Fierens, & Delcour, 2015).

On the other hand, protein is traded as dried gluten known as vital wheat gluten, which recovers its visco-elastic functional properties upon rehydration (Day et al., 2006).

Traditional wet milling of wheat utilizes flour that is first converted into a dough, which is then kneaded while being washed in excess water. The washing of the kneaded dough removes starch and water soluble material (mostly sugar and protein). The remaining dough mass is primarily gluten protein. This simple method is often referred to as the Martin method (Van Der Borght, Goesaert, Veraverbeke, & Delcour, 2005).

In North America, the need for industrial wheat starch for laundry purposes led to commercial wet milling. Gluten produced was initially discarded but later was incorporated as vital wheat gluten into many food products (Day et al., 2006). Kempf, & Röhrmann (1989) described fifteen wet milling processes, starting with either wheat kernels or flour. However, only five of those processes, being the Martin (dough-washing), the Batter, the Alfa-Laval/Raisio, the Hydrocyclone, and the High-Pressure Disintegration (HD), have been industrially employed, and all commercial processes start with wheat flour rather than wheat kernels (Sayaslan, 2004). Currently, the commercially utilized processes in North America are the Martin, Alfa-Laval/Raisio, and Hydrocyclone, while the High-Pressure Disintegration (HD) process is used in Europe (Sayaslan, 2004).

DON represents a food safety concern as it has toxicity towards humans and ruminant and non-ruminant animals. Some of the negative effects typical of DON mycotoxicosis in humans are diarrhea, nausea, vomiting, abdominal pain, and fever; whereas feed refusal, weakness, and vomiting can be seen in animals (Wegulo, 2012). Previous research (chapter 3) showed that little or no DON was found in starch or gluten isolated from farina/semolina and shorts after wet milling. In that research the Martin process was used. The research reported here compared the Martin, medium shear, and high shear wet milling processes on their ability to remove DON from farina or semolina. It is unknown what effect wet milling procedure might have on the removal of DON. Therefore, the aim of the research was to compare the effectiveness of three laboratory scale wet milling processes to remove DON from wheat.

4.3. Materials and Methods

4.3.1. Materials

Durum wheat samples were obtained during the 2015 harvest season; samples came directly from grower fields, farm bins, and local elevators. They were collected as part of the annual durum crop quality survey by the Montana and North Dakota National Agricultural Statistics Service offices. Samples were stored in sealed moisture-proof plastic bags. Two bulk samples of durum wheat were made. The first sample was a blend of three durum wheat samples that contained DON. Both bulk samples contained 3 kg of wheat. Similarly, two samples of Hard Red Spring Wheat were made. The first sample contained HRSW cultivars that contained DON. These cultivars were grown in 2016 at the North Dakota State University Langdon Research Extension Center (Langdon, ND). The second sample contained grain from a cultivar grown in 2016 at North Dakota State University Williston Research Extension Center (Williston, ND) that had no detectable DON. Each sample was cleaned and divided into three 1 kg samples, which were further divided into 250 g subsamples and stored at -18°C until needed.

4.3.2. Grain and Farina/Semolina Proximate Analysis

Grain moisture content, ash content, protein content, total starch, and falling number were determined using AACCI Approved Methods 44-15.02, 08-01.01, 46-30.01, 76-13.01, and 56-81.03, respectively. Additionally, 1000-kernel weight was determined based on the number of kernels in ten grams of clean grain as described in Chapter 3. Farina/semolina total starch content and protein content were determined using AACCI Approved Methods 76-13.01 and 46-30.01, respectively.

4.3.3. Dry Milling

Samples were cleaned by passing through a Carter – Day dockage tester. Cleaned samples (150 g) were tempered to 15.5% moisture content 24 h before milling. Samples were milled using a Brabender Quadrumat Jr roller mill (C.W. Brabender Instruments, Inc. South Hackensack, NJ, USA). Three milling fractions were collected: bran, shorts, and semolina (durum) or farina (HRSW) for DON content assessment. Only semolina and farina were collected for the wet milling process.

4.3.4. Wet Milling

Three different wet milling methods were utilized in the present research. The first one resembled the traditional Martin wet milling method (Figure 4.01), based on the AACC International Approved

Method (38-12.02) for determining wet gluten content. The method was followed, with slight modifications. Two sets of 10 g samples were washed with ≈300 mL of distilled water to remove starch and soluble material from the developed gluten matrix using a Glutomatic 2200 (Perten Instruments, PerkinElmer Inc., MA, USA). Starch was separated from water soluble material by centrifugation at 2500 x *g* for 15 min. The other two wet milling methods were performed according to the moderately sheared dough-water dispersion (medium shear) (Figure 4.02) and highly shear flour-water dispersion (high shear) (Figure 4.03) as described by Sayaslan, Seib, & Chung (2012), with a modification in the starting material of HS-FWD being of 35 g. The obtained wet gluten and starch from the three different wet milling methods were then dried overnight at 45°C in a convection air oven and weighed. In addition, the residual water from the washings for each sample was combined and freeze-dried.

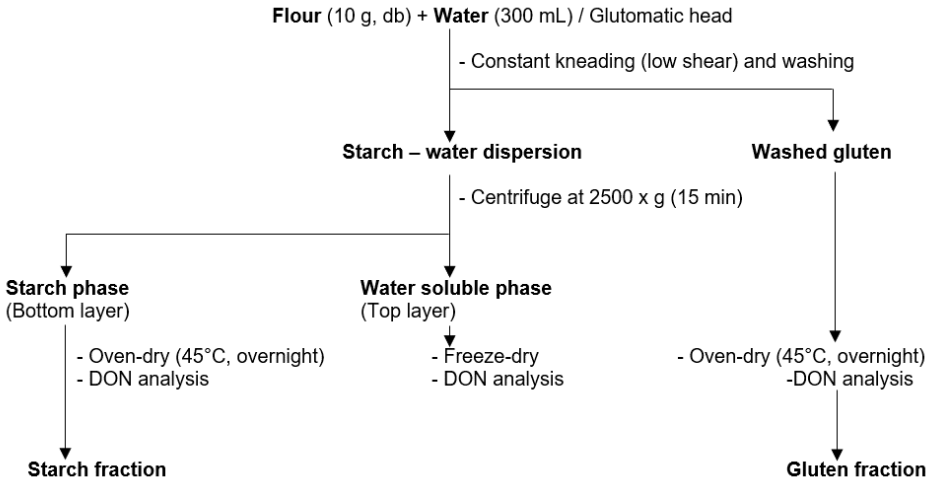

Figure 4.01. Martin wet milling process diagram.

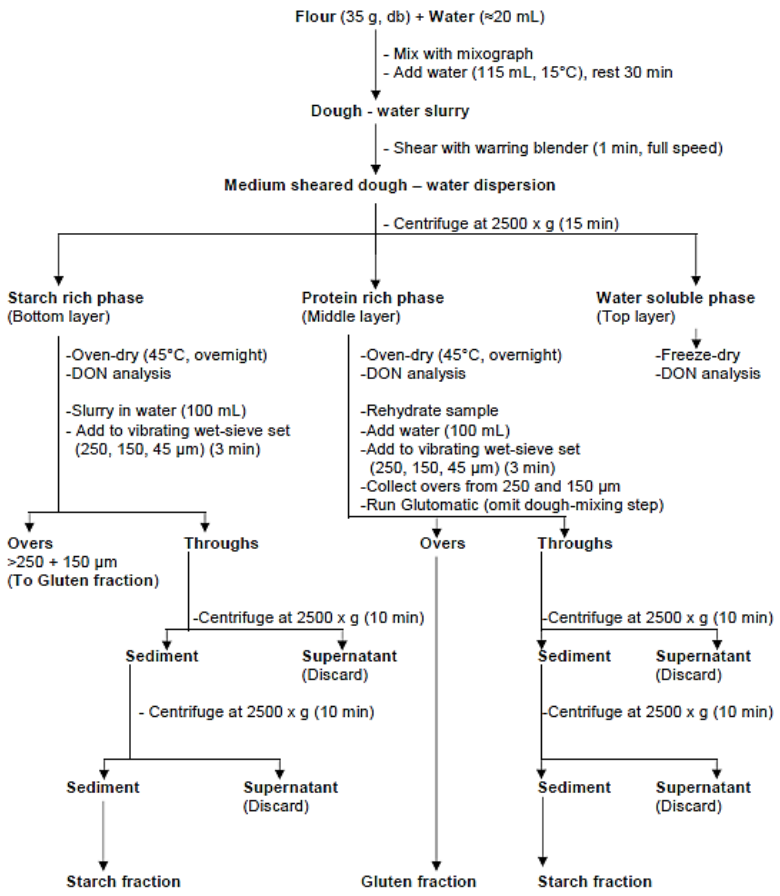

Figure 4.02. Medium shear wet milling process diagram.

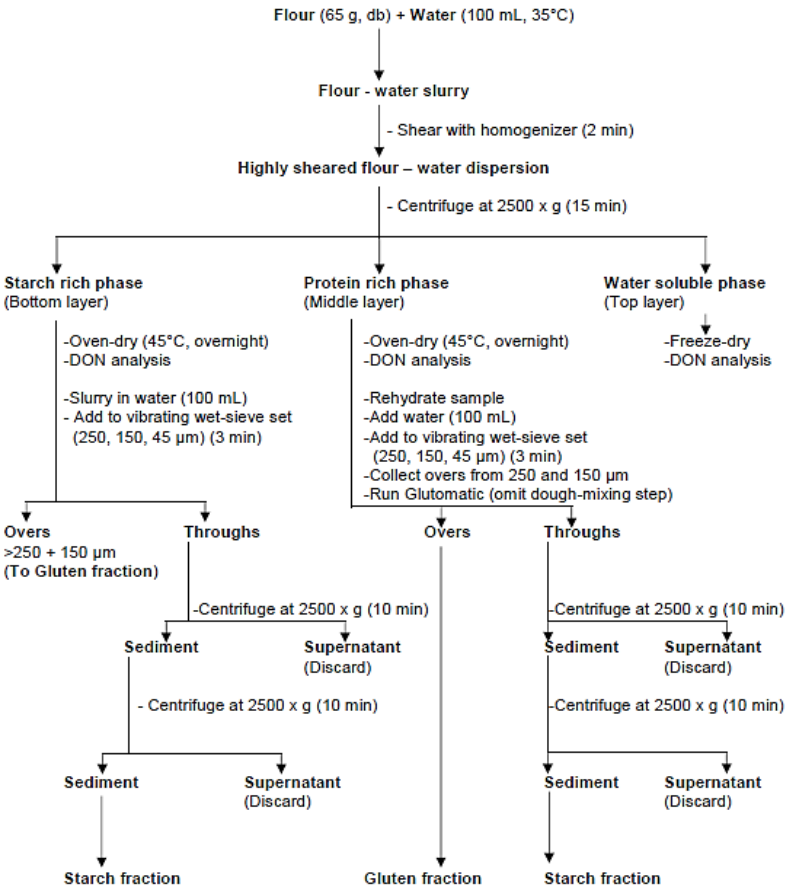

Figure 4.03. High shear wet milling process diagram.

4.3.5. DON Analysis

Mycotoxin assessment was performed by determining DON levels in whole wheat flour, the dry milled fractions (farina/semolina, shorts and bran) and the wet milled fractions (gluten, starch and water solubles). DON was determined by gas chromatography with electron capture detection (GC-ECD) as described by Tacke, & Casper (1996) and Simsek, Burgess, Whitney, Gu, & Qian (2012), with a modification of the extraction method using 1 g sample and 8 mL of acetonitrile-water (84:16). The samples were analyzed with an Agilent (Santa Clara, CA, USA) 6890 GC with cool on-column inlet, DB-5 column and ECD detector. Helium was used as the carrier gas and argon-methane (95:5) was used as the makeup gas. This method had a limit of quantification of 0.2-40 mg/kg.

4.3.6. Statistical Analysis

The experimental design was a randomized complete block with two factorial arrangement and four replications. HRSW and DW samples were treated as separate experiments. The first factor was the wet milling method (three methods: Martin, medium shear, high shear) and the second factor was mycotoxin contamination (two levels: with and without DON). Data were subjected to analysis of variance using the 'MIXED' procedure in the Statistical Analysis System 9.4 (SAS Institute, Cary, NC, USA). Treatment means were separated by Fisher's Protected Least Significant Different (LSD) at $P<0.05$. Correlation analysis was conducted using 'CORR' procedure in the Statistical Analysis System 9.4 (SAS Institute, Cary, NC, USA).

4.4. Results and Discussion

4.4.1. Characterization of Starting Material

Mean values for the DON levels in whole wheat flour (WWF) and grain proximate analysis are presented in Table 4.01. For HRSW and DW, sample 1 contained high levels of DON, 6.0 mg/kg and 11.6 mg/kg for HRSW 1 and DW 1, respectively. On the other hand, HRSW 2 and DW 2 wer the controls and had no detectable DON levels.

The 1000-kernel weight, falling number and protein, starch, and ash content values of HRSW and DW samples are typical of wheat grown in the Northern Plains of US. as evidenced by their similarity to their five-year crop average (Regional Quality Reports, 2017), respectively. Thus these grain samples (even those with high DON levels) had acceptable quality.

It is well-known that early infections by *Fusarium* greatly reduce 1000-kernel weight due to the development of small, shriveled, and lightweight kernels (Del Ponte, Fernandes, & Bergstrom, 2007; Matthäus et al., 2011; Schwarz, Schwarz, Zhou, Prom, & Steffenson, 2001). The acceptable grain quality suggests that *Fusarium* infection of HRSW and DW samples occurred late in kernel development, since they did not suffer a dramatic weight decrease due to infection.

Differences in protein content, ash content, and falling number that did occur between samples with and without DON are attributed primarily to cultivar and/or growing environment and not to *Fusarium* infection. This is supported by the lack of differences in starch contents and to the high falling numbers that would indicate that any hydrolytic enzymes that the *Fusarium* infection might have released was not detected by decline in starch or in falling number. Falling numbers above 400 sec indicate low α-amylase activity. Had *Fusarium* infection released α-amylase the falling number would be expected to be much lower. For example, Wang, Pawelzik, Weinert, Zhao, & Wolf (2008) reported that wheat infected by *Fusarium* had falling number of 275 sec, claiming a significant positive relationship between the degree of *Fusarium* infection and fungal α-amylase, affecting falling number.

Table 4.01. DON levels detected and proximate analysis (mean ±SD) of HRSW and DW samples.

Sample	DON WWF		1000-kernel weight		Protein [c]		Total starch		Ash		Falling number	
	----- mg/kg -----		--------- g ---------		---------- % ----------						-------- sec ------	
HRSW [a] 1	6.0	±0.03	28.5 b	±1.02	13.9 a	±0.07	50.59 a	±1.71	1.58 a	±0.02	412 b	±28.53
HRSW 2	ND [d]		32.6 a	±0.38	14.2 a	±0.12	51.05 a	±1.66	1.49 b	±0.03	522 a	±7.57
5-year Avg.	0.20		31.3		14.0		-		1.52		388	
DW [b] 1	11.6	±0.39	38.8 b	±0.82	13.5 b	±0.16	51.85 a	±1.59	1.73 a	±0.04	460 a	±6.04
DW 2	ND		46.2 a	±0.26	14.4 a	±0.10	51.61 a	±0.67	1.39 b	±0.07	487 a	±30.43
5-year Avg.	1.10		39.6		13.6		-		1.59		380	

Means with the same letter within columns for the same wheat type are not significantly different ($P<0.05$).

[a] HRSW: Hard red spring wheat.

[b] DW: Durum wheat.

[c] 12% moisture basis.

[d] ND: Below the limit of quantification (<0.2 mg/kg).

4.4.2. Dry Milling and DON Distributions in Dry Milling Fractions

As expected, fraction level was farina/semolina > bran > shorts for both HRSW and DW samples (Table 4.02). HRSW and DW samples did mill differently. HRSW produced more farina and less shorts than did DW samples. The bran fractions were similar for both HRSW and DW. These results indicate that it was more difficult to separate bran from endosperm in DW than in HRSW. HRSW yielded higher amount of farina and shorts fractions in the control than in the infected grain. Infected grain resulted in a large bran fraction. Whereas DW had larger semolina yield and smaller shorts fraction with DON infected grain compared to the control. Bran fractions were similar within DW samples, while HRSW sample with DON yielded a larger amount of bran than the control sample.

DON was found in all three mill fractions of HRSW 1 and DW 1. The mycotoxin levels and distribution in each dry milled fraction are shown in Table 4.03. All fractions except for farina (3 mg/kg DON) contained DON levels above 5 mg/kg which exceed the FDA advisory levels for consumption by humans and non-ruminant animals. The DON levels followed the common trend of bran> shorts> farina/semolina DON contamination as reported in previous research (Bullerman, & Bianchini, 2007; Thammawong et al., 2011; Tibola, Cunha Fernandes, Guarienti, & Nicolau, 2015), where the highest amounts of mycotoxin are found in fractions less likely for human consumption, such as shorts and bran (Bullerman, & Bianchini, 2007; Cheli, Pinotti, Rossi, & Dell'Orto, 2013). For HRSW, 34% of DON occurred in farina, 12% in shorts and 54% in bran. While for DW, 42% of DON was found in semolina, 15% in shorts, and 45% in bran. These results are in agreement with the results reported in Chapter 3 and with those reported by Cheli et al. (2013). They reported that, typically, the concentration of mycotoxins in either flour or semolina can be up to 50%, depending on the wheat type and variety, degree of *Fusarium* infection and mycotoxin transfer to kernel inner parts.

Table 4.02. Dry milling yields for HRSW and DW samples.

Sample	Farina/Semolina fraction	Shorts fraction	Bran fraction
	---------- % ----------		
HRSW 1	61.3 b	8.2 b	30.5 a
HRSW 2	64.5 a	9.2 a	26.3 b
DW 1	54.0 a	17.8 b	28.3 a
DW 2	51.4 b	20.1 a	28.5 a

Means with the same letter within columns for the same wheat type are not significantly different (P<0.05).

Table 4.03. DON levels (mean ±SD) detected in WWF, farina/semolina, shorts and bran of HRSW and DW samples.

Sample	DON WWF	DON farina/ semolina	DON shorts	DON bran	DON farina/ semolina [a]	DON shorts [b]	DON bran [c]
	---------- mg/kg ----------				---------- % ----------		
HRSW 1	6.0 ±0.70	3.0 ±0.54	7.6 ±1.58	9.5 ±1.08	34	12	54
HRSW 2	ND[d]	ND	ND	ND	-	-	-
DW 1	11.9 ±1.32	8.8 ±0.78	7.8 ±1.04	17.5 ±1.85	42	14	44
DW 2	ND	ND	ND	ND	-	-	-

Means with the same letter within columns for the same wheat type are not significantly different (P<0.05).
[a] DON farina/semolina recovery (%)= [Farina/semolina yield (%) x DON level (mg/kg)] / [WWF DON level (mg/kg)] x 100.
[b] DON shorts recovery (%)= [Shorts yield (%) x DON level (mg/kg)] / [WWF DON level (mg/kg)] x 100.
[c] DON bran recovery (%)= [Bran yield (%) x DON level (mg/kg)] / [WWF DON level (mg/kg)] x 100.
[d] ND: Below the limit of quantification (<0.2 mg/kg).

4.4.3. Farina / Semolina Wet Milling

Starch content and starch extracted during wet milling HRSW and DW samples are reported in Table 4.04. The total amount of starch in HRSW and DW samples were similar, ranging from 61.5 to 61.6% for HRSW and 59.6 to 60.8% for DW. The relative effectiveness of wet milling process was similar for HRSW and DW samples. For example, the amount of crude starch extracted from HRSW and DW samples was greatest with the Martin process, intermediate with medium shear, and least with high shear wet milling process. Extracted starch purity differed significantly among samples. Overall, purity tended to be greatest with medium shear (80.1-82.2%), intermediate with Martin (77.6-80.0%), and least with high shear (72.0-77.1%) wet milling processes. The large differences among wet milling processes in crude starch and net starch yields reflect the number of purification steps. In the Martin wet milling process, all starch was recovered in a single fraction and was not subjected to further purification steps, as seen in Figure 4.04, whereas for the medium and high shear wet milling processes after their first centrifugation step the continuous dense bottom stream was collected from the base of the containers and was subjected to further purification, with loss of starch occurring with each step. Loss of B-granules during wet milling has been reported to result in low starch yields. In other words, during medium and high shear processes gluten might be entrapping B-granules preventing them from being released. In this regard, Maningat et al. (2009) stated that from 23 to 50 % of starch weight percent corresponded to B-granules.

This suggests that the recovered starch fraction in the study would be mainly composed by A-starch granules, explaining why the net yields ranged from 23 to 25% for HRSW during medium shear and from 18 to 27% for HRSW during high shear, while from 26 to 31% for DW during medium shear and from 12 to 21% for DW during high shear. To illustrate this, Figure 4.05 shows an SEM of the starch from HRSW DON contaminated sample and HRSW control sample extracted by the three tested wet milling methods. The traditional Martin process had a large number of B-starch granules which formed clumps due to their higher proportion when compared to the other two wet milling methods. SEM of medium shear and high shear show progressively fewer B-granules. Steeneken, & Helmens (2009) reported that the occurrence of B-starch granules does not depend on the starting material but on the way the starch-gluten separation is achieved. As seen from the obtained net yields and recoveries, the efficacy of starch extraction from the medium shear process was higher than that from high shear process.

For both HRSW and DW, the DON contaminated sample yielded more starch. A higher starch recovery was seen in the DON contaminated sample from the high shear process compared to the DON free sample for both wheat species. This slight improvement in the efficacy of the high shear process may be due to an alteration on the coalescence of proteins physically entrapping the starch granules (Stenvert, & Kingswood, 1977) that lead to a better wet milling performance. To illustrate this, in Figure 4.06 is evident the difference in gluten coalescence from a HRSW DON contaminated sample with the different wet milling processes, and the amount of starch that came off during the first centrifugation with the gluten fraction, which underwent further purification steps. In other words, high shear sample has more starch associated with it than does medium shear sample, while the Martin wet milled sample has very little visual starch associated with the gluten.

DON levels on the extracted starch fractions were below the limits of quantification, which may be due to the removal of the majority of the solubles during the washing steps. The obtained results agree with earlier work, where corn contaminated with water-soluble mycotoxins, like DON and nivalenol (NIV), was successfully wet milled yielding a mycotoxin free starch fraction (Bennett, & Anderson, 1978; Lauren, & Ringrose, 1997; Okeke et al., 2017). Additionally, the results also agree with Chapter 3, having all obtained starch fractions DON levels below the limit of quantification.

Table 4.04. Effect of wet milling process on the content, extraction properties, and DON content of starch obtained by wet milling farina/semolina from HRSW and DW samples.

Sample	Farina / Semolina total starch	Crude starch yield [a]	Extracted starch purity	Net starch yield [b]	Starch recovery [c]	DON level in extracted starch
	%	---------- % ----------				mg/kg
HRSW 1						
Martin	61.5 a	65.1 a	77.6 b	50.5 a	82.2 a	ND [d]
Medium shear	61.5 a	28.4 bc	80.4 ab	22.8 bc	37.2 bc	ND
High shear	61.5 a	34.8 b	76.6 b	26.5 b	44.0 b	ND
HRSW 2						
Martin	61.6 a	67.8 a	80.0 ab	54.2 a	88.0 a	ND
Medium shear	61.6 a	29.8 bc	82.2 a	24.5 bc	39.9 b	ND
High shear	61.6 a	24.9 c	72.0 c	17.9 c	29.0 c	ND
DW 1						
Martin	60.8 a	67.8 a	79.9 ab	54.2 a	89.2 a	ND
Medium shear	60.8 a	39.1 b	80.1 ab	31.3 b	51.5 b	ND
High shear	60.8 a	27.5 d	77.1 ab	21.2 d	35.1 d	ND
DW 2						
Martin	59.6 a	68.5 a	79.0 ab	54.1 a	90.9 a	ND
Medium shear	59.6 a	31.6 c	81.8 a	25.8 c	43.4 c	ND
High shear	59.6 a	15.0 e	76.4 b	11.5 e	19.3 e	ND

Means with the same letter within columns for the same wheat type are not significantly different (P<0.05).
[a] Crude starch yield (%)= [Extracted starch (g, dm) / Amount of wet milled farina/semolina (g, dm)] x 100.
[b] Net starch yield (%)= {[Extracted starch (g, dm) / Amount of wet milled farina/semolina (g, dm)] x 100} Starch purity (%).
[c] Starch recovery (%)= [Extracted starch (g, dm) x Starch purity (%)] / [Amount of wet milled farina/semolina (g, dm) x Starch content (%)] x 100.
[d] ND: Below the limit of quantification (<0.2 mg/kg).

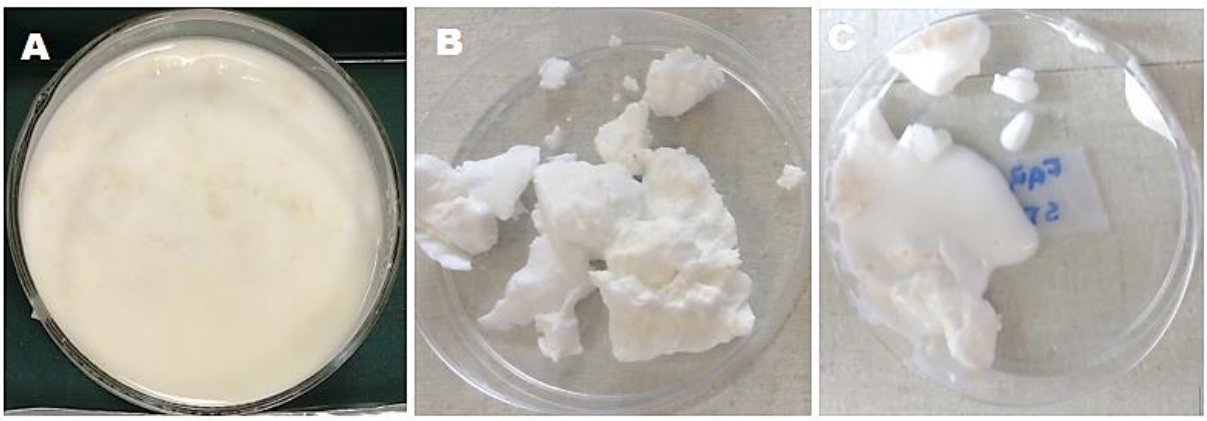

Figure 4.04. Extracted starch before drying step of HRSW DON contaminated sample extracted by A) Martin; B) medium shear; and C) high shear processes.

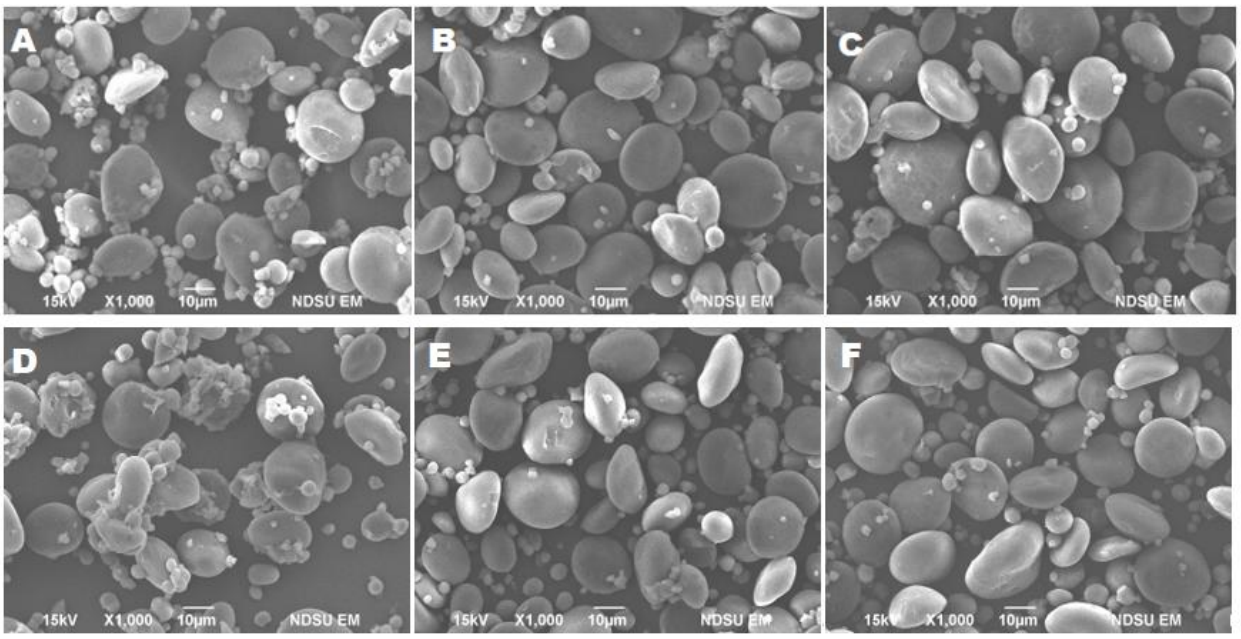

Figure 4.05. Scanning Electron Micrograph (SEM) of HRSW DON contaminated sample extracted by A) Martin; B) medium shear; and C) high shear processes; and HRSW control sample extracted by D) Martin; E) medium shear; and F) high shear processes.

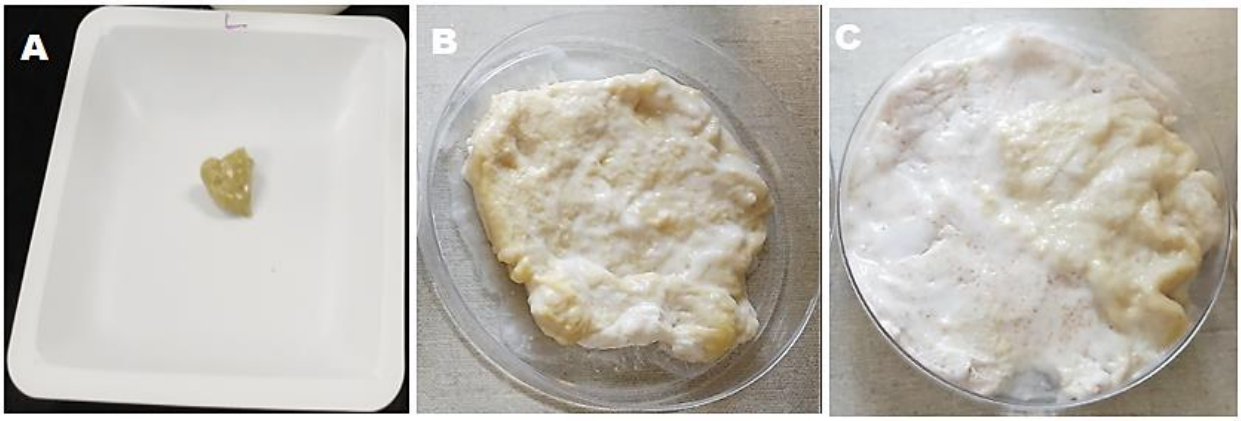

Figure 4.06. Extracted gluten HRSW DON contaminated sample extracted by A) Martin (before drying step); B) medium shear (before further purification); and C) high shear processes (before further purification).

Table 4.05 displays extracted gluten results with and without DON using different wet milling processes. Overall, gluten extraction, purity, and recovery were not greatly affected by wheat species but were affected by wet milling process and by the presence of DON in the sample. For a given sample, the amount of extracted crude gluten was greatest with medium shear, intermediate with Martin, and least with high shear processes. Regardless of sample, the purity of the extracted gluten was greatest with the

Martin process and tended to be similarly lower for the medium and high shear processes. Extracted gluten purity with Martin process seemed to be similar for HRSW and DW and for samples with and without DON and exceeded the minimum standard for commercial gluten (>73%). The presence of DON seemed to affect wet milling using medium shear or high shear processes but not the Martin process. Purity of gluten extracted using medium and high shear processes were similar for HRSW and DW but was greater in DON containing samples. Medium and high shear processing of samples without DON resulted in low purity (40.7-48.3%) which is below the level that is commercially acceptable.

The greatest net yield (14.5%) and percent gluten recovery (99.9%) occurred using the medium shear process to wet milling either HRSW or DW samples that contained DON. For samples without DON, net yield and percent gluten recovery was similar for Martin and medium shear processes. The percent gluten recovery by the Martin process was fairly consistent (66.6-69.1%) across wheat species and samples with and without DON. Overall the net yield and percent gluten recovery was much less with the high shear than with the medium shear or Martin processes.

With the traditional Martin wet milling, gluten was formed due to constant kneading and washing actions. With the medium and high shear wet milling processes, gluten agglomeration varied, as seen in Figure 4.06, and the gluten fraction emerged together with B-starch fraction containing a bulk of small and damaged granules (Steeneken, & Helmens, 2009), which was the gluten rich fraction subjected to further washings on the vibrating screens and Glutomatic.

In addition, Steeneken, & Helmens (2009) reported a gluten purity range of 56 to 77% by wet milling using only water. Sayaslan et al. (2006) reported gluten fractions with purities ranging from 70 to 82% obtained from wet milling waxy wheat flour with water by dough-washing and medium shear processes. In this regard, the obtained results are within the range of purities reported from previous research. However, the coincident low purities from both HRSW and DW DON free samples in medium and high shear processes suggest more difficulty to remove the adhered starch from these gluten samples. On the other hand, the low net yields and recoveries obtained from HRSW and DW DON free samples wet milled with the high shear process suggest a lack of satisfactory gluten agglomeration so as to have an adequate wash and recovery of gluten proteins.

In addition, similarly as in the extracted starch fraction, gluten fractions denoted DON levels below the limit of quantification. These results agree with Lauren, & Ringrose (1997), which stated that the concentration of mycotoxins in obtained gluten from wet milling contaminated corn was very low when compared to the original material mycotoxin levels, claiming relatively toxin free fractions. However, the same authors also reported that some of the samples yielded gluten fractions with low levels of mycotoxin contamination, as it happened in Chapter 3. Therefore, unlike starch fraction, special attention has to be given to the complete and satisfactory removal of mycotoxins, like DON, from gluten fraction.

Table 4.05. Effect of wet milling process on the content, extraction properties, and DON content of gluten obtained by wet milling farina/semolina from HRSW and DW samples.

Sample	Farina / Semolina total protein	Crude gluten yield [a]	Extracted gluten purity	Net gluten yield [b]	Gluten recovery [c]	DON level in extracted gluten
	%	---------- % ----------				mg/kg
HRSW 1						
Martin	14.5 a	11.9 c	84.6 b	10.1 b	69.1 b	ND [d]
Medium shear	14.4 a	19.8 b	73.4 d	14.5 a	99.9 a	ND
High shear	14.3 a	7.5 d	78.7 c	5.9 d	41.3 d	ND
HRSW 2						
Martin	15.3 a	11.6 c	87.7 a	10.2 b	66.6 bc	ND
Medium shear	15.2 a	22.9 a	40.7 e	9.3 c	61.1c	ND
High shear	15.1 a	7.0 d	42.4 e	3.0 e	19.6 e	ND
DW 1						
Martin	14.2 a	11.5 c	83.5 a	9.6 b	67.3 b	ND
Medium shear	14.3 a	18.2 b	79.8 a	14.5 a	99.9 a	ND
High shear	13.9 a	2.4 d	70.1 b	1.7 c	12.2 c	ND
DW 2						
Martin	15.6 a	12.7 c	83.1 a	10.6 b	68.0 b	ND
Medium shear	15.6 a	22.4 a	48.3 c	10.8 b	69.7 b	ND
High shear	15.5 a	3.3 d	47.3 c	1.5 c	9.9 c	ND

Means with the same letter within columns for the same wheat type are not significantly different (P<0.05).
[a] Crude gluten yield (%)= [Extracted gluten (g, dm) / Amount of wet milled farina/semolina (g, dm)] x 100.
[b] Net gluten yield (%)= {[Extracted gluten (g, dm) / Amount of wet milled farina/semolina (g, dm)] x 100} Gluten purity (%).
[c] Gluten recovery (%)= [Extracted gluten (g, dm) x Gluten purity (%)] / [Amount of wet milled farina/semolina (g, dm) x Protein content (%)] x 100.
[d] ND: Below the limit of quantification (<0.2 mg/kg).

The obtained starch and gluten fractions exhibited DON levels below the limit of quantification, therefore high levels of DON were expected to be found in the water soluble fraction. In this regard, Table

4.06 displays the traits related to the water soluble fraction from DON infected samples and control samples arranged by wet milling process for both HRSW and DW. Special attention has to be drawn since the collected water for mycotoxin analysis corresponded to the water obtained from the first stage of washings, where the first starch rich and gluten rich fractions were collected. Further water fractions yielded by the medium and high shear processes were not analyzed due to the lack of space and facilities for storage and timely freeze-drying. From the observed results it is evident that the high DON levels found in the water soluble fraction were aided by the mycotoxin high solubility, in agreement with mycotoxin contaminated corn wet milling research (Bennett, & Anderson, 1978; Bennett, Richard, & Eckhoff, 1996; Lauren, & Ringrose, 1997) and chapter 3 of this thesis. Regarding HRSW, even when most of DON was collected using the Martin and high shear processes, an unsatisfactory 75% of DON was accounted with the medium shear process. However, this might suggest that the rest of the mycotoxin was found in the further unanalyzed water soluble fraction. On the other hand, regarding DW samples, we have to consider that the starting level of DON in semolina (11.92 mg/kg) was considerably higher than that one in farina (5.96 mg/kg), therefore a higher amount of DON should be expected in the water soluble fraction. Even when the DON levels obtained in DW water solubles fraction were considerably high, they were not enough to account recoveries of more than 56%, regardless the wet milling process utilized. Once again, we are assuming that this is an under estimation of the real amounts of DON concentrated in the freeze-dried water soluble fractions, since for obtaining at least 90% of DON recovery, more than 200 mg/kg should have been found in the Martin, medium shear and high shear water soluble fractions. Therefore, these excessively high DON concentrations would not be possible to be measured accurately with our GC-ECD equipment without diluting the sample, which in this case was not possible due to the small amount of freeze-dried sample available (≈1 g). The DW DON levels results might indicate an under estimation of the real amount of mycotoxin recovered in the water since 90 mg/kg were measured by the GC-ECD equipment, which is calibrated for up to 40 mg/kg, so the higher the amount of DON that is wanted to measure, the more inexact the result will be. In this regard, for accounting at least 90 % of the mycotoxin a result of 235 mg/kg of DON would have been expected, which is very high and almost impossible to obtain with the utilized equipment.

Table 4.06. Effect of wet milling process on content, extraction properties, and DON content of water solubles obtained by wet milling farina/semolina from HRSW and DW samples.

Sample	Crude yield water Soluble fractions [a]	Water solubles DON level	Water solubles DON recovery [b]
	%	mg/kg	%
HRSW 1			
Martin	4.1 a	76.45 a	103
Medium shear	3.6 b	62.69 b	75
High shear	3.6 b	78.81 a	93
HRSW 2			
Martin	4.1 a	ND [c]	-
Medium shear	3.5 c	ND	-
High shear	3.5 c	ND	-
DW 1			
Martin	5.6 a	87.24 b	56
Medium shear	3.9 c	92.37 a	41
High shear	4.3 c	91.20 a	45
DW 2			
Martin	5.0 b	ND	-
Medium shear	4.4 bc	ND	-
High shear	4.3 c	ND	-

Means with the same letter within columns for the same wheat type are not significantly different (P<0.05).
[a] Crude yield water soluble fractions (%)= [Extracted water solubles (g, dm) / Amount of wet milled farina/semolina (g, dm)] x 100.
[b] Water solubles DON recovery (%)= [Water solubles yield (%) x DON level (mg/kg)] / [Farina/semolina DON level (mg/kg)] x 100.
[c] ND: Below the limit of quantification (<0.2 mg/kg).

4.5. Conclusions

In general, the three tested wet milling methods, Martin, medium shear, and high shear were effective in the removal of DON from starch and gluten fractions for both wheat types. DON levels of wet milled products, starch and gluten, were below the limit of quantification. More than 90% of DON levels were recovered from HRSW samples with Martin and high shear process, while only more than 40% of DON was recovered from DW samples. The best starch and gluten recoveries were obtained with Martin process. The high shear process had the lowest starch and gluten recoveries. Wet milling of DON contaminated grain can be considered a promising alternative for farmers and industrial starch and gluten manufacturers.

4.6. References

AACC International. *Approved methods of analysis* (11th Ed.). Methods, 08-01.01, 44-15.02, 46-30.01, 56-81.03, and 76-13.01. Available online only. St. Paul, MN: AACCI.

Anonymous. (2017). U.S. Hard red spring wheat Regional Quality Report 16. pp. 36.

Anonymous. (2017). U.S. Durum wheat Regional Quality Report 16. pp. 24.

Bennett, G. A., & Anderson, R. A. (1978). Distribution of aflatoxin and/or zearalenone in wet-milled corn products: A review. *Journal of Agricultural and Food Chemistry*, *26*, 1055-1060.

Bennett, G. A., Rcihard, J. L., & Eckhoff, S. R. (1996). Distribution of fumonisins in food and feed products prepared from contaminated corn. In L. S. Jackson, J. W. DeVries, and L. B. Bullerman (Eds.), *Fumonisins in food* (pp. 317-322). New York: Plenum Press.

Bullerman, L. B., & Bianchini, A. (2007). Stability of mycotoxins during food processing. *International Journal of Food Microbiology*, *119*, 140-146.

Cheli, F., Pinotti, L., Rossi, L., & Dell'Orto, V. (2013). Effect of milling procedures on mycotoxin distribution in wheat fractions: A review. *Journal of Food Science and Technology*, *54*, 307-314.

Day, L., Augustin, M. A., Batey, I. L., & Wrigley, C. W. (2006). Wheat-gluten uses and industry needs. *Trends in Food Science & Technology*, *17*, 82-90.

Del Ponte, E. M., Fernandes, J. M., & Bergstrom, G. C. (2007). Influence of growth stage on Fusarium Head Blight and deoxynivalenol production in wheat. *Journal of Phytopathology*, *155*, 577-581.

Dexter, J. E., Marchylo, B. A., Clear, R. M., & Clarke, J. M. (1997). Effect of Fusarium Head Blight on semolina milling and pasta-making quality of durum wheat. *Cereal Chemistry*, *74*, 519-525.

FAO. (2018). http://www.fao.org/faostat/en/ Food and Agriculture Organization of the United Nations Statistics.

Jones, H. D. (2005). Wheat transformation: Current technology and applications to grain development and composition. *Journal of Cereal Science*, *41*, 137-147.

Kempf, W., & Röhrmann, C. (1989). Process for the industrial production of wheat starch form whole wheat. In Y. Pomeranz (Ed.), *Wheat is Unique: Structure, Composition, Processing, End-use Properties, and Products* (pp. 521-540). St. Paul: AACC International.

Khaneghah, A. M., Martins, L. M., von Hertwig, A. M., Bertoldo, R., & Sant'Anna, A. S. (2018). Deoxynivalenol and its masked forms: Characteristics, incidence, control and fate during wheat and wheat based products processing – A review. *Trends in Food Science & Technology*, *71*, 13-24.

Lauren, D. R., & Ringrose, M. A. (1997). Determination of the fate of three Fusarium mycotoxin through wet-milling of maize using an improved HPLC analytical technique. *Food Additives & Contaminants*, *14*, 435-443.

Maningat, C. C., Seib, P. A., Bassi, S. D., Woo, K. S., & Lasater, G. D. (2009). Wheat starch: Production, properties, modification and uses. In J. BeMiller, and R. Whistler (Eds.), *Starch chemistry and technology* (pp. 441–510). USA: Elsevier Inc.

Matthäus, K., Dänicke, S., Vahjen, W., Simon, O., Wang, J., Valenta, H., Meyer, K., Strumpf, A., Ziesenib, H., & Flachowsky, G. (2011). Progression of mycotoxin and nutrient concentrations in wheat after inoculation with Fusarium culmorum. *Archives of Animal Nutrition*, *58*, 19-35.

Meyer, D., Weipert, D., & Mielke, H. (1986). Influencing the quality of wheat by infecting with Fusarium culmorum. *Getreide, Mehl und Brot*, *40*, 35-39.

Okeke, C. A., Ezekiel, C. N., Sulyok, M., Ogunremi, O. R., Ezemagu, C. O., Sarkanj, B., Warth, B., & Krska, R. (2017). Traditional processing impacts mycotoxin levels and nutritional value of ogi – A maize-based complementary food. *Food Control*, *86*, 224-233.

Sayaslan, A. (2004). Wet-milling of wheat flour: Industrial processes and small-scale test methods. *Journal of Food Science and Technology*, *37*, 499-515.

Sayaslan, A., Seib, P. A., & Chung, O. K. (2006). Wet-milling properties of waxy wheat flours by two laboratory methods. *Journal of Food Engineering*, *72*, 167-178.

Sayaslan, A., Seib, P. A., & Chung, O. K. (2012). A bench-scale high-shear wet-milling test for wheat flour. *Journal of Food Engineering*, *111*, 305-317.

Schwarz, P. B., Schwarz, J. G., Zhou, A., Prom, L. K., & Steffenson, B. J. (2001). Effect of Fusarium graminearum and Fusarium poae infection on barley and malt quality. *Monatsschrift fur Brauwissenschaft*, *3*, 55-63.

Simsek, S., Burges, K., Whitney, K. L., Gu, Y., & Qian, S. Y. (2012). Analysis of deoxynivalenol and deoxynivalenol-3-glucoside in wheat. *Food Control*, *26*, 287-292.

Steeneken, P. A. M., & Helmens, H. J. (2009). Laboratory-scale dry/wet-milling process for the extraction of starch and gluten from wheat. *Starch-Stärke*, *61*, 389-397.

Stenvert, N. L., & Kingswood, K. (1977). The influence of the physical structure of the protein matrix on wheat hardness. *Journal of the Science of Food and Agriculture*, *28*, 11-19.

Tacke, B. K., & Casper, H. H. (1996). Determination of deoxynivalenol in wheat, barley, and malt by column cleanup and gas chromatography with electron capture detection. *Journal of AOAC International*, *79*, 472-475.

Thammawong, M., Okadome, H., Shiina, T., Nakagawa, H., Nagashima, H., Nakajima, T., & Kushiro, M. (2011). Distinct distribution of deoxynivalenol, nivalenol, and ergosterol in Fusarium-infected Japanese soft red winter wheat milling fractions. *Mycopathologia*, *172*, 323-330.

Tibola, C. S., Cunha Fernandes, J. M., Guarienti, E. M., & Nicolau, M. (2015). Distribution of Fusarium mycotoxins in wheat milling process. *Food Control*, *53*, 91-95.

Van Der Borght, A., Goesaert, H., Veraverbeke, W. S., & Delcour, J. A. (2005). Fractionation of wheat and wheat flour into starch and gluten: overview of the main processes and the factors involved. *Journal of Cereal Science*, *41*, 221-237.

Wang, D., & Chung, D. S. (2002). Study of a small-scale laboratory wet-milling procedure for wheat. *Cereal Chemistry*, *79*, 115-119.

Wang, J., Pawelzik, E., Weinert, J., & Wolf, G. A. (2005). Impact of Fusarium culmorum on the polysaccharides of wheat flour. *Journal of Agricultural and Food Chemistry*, *53*, 5818-5823.

Wang, J., Pawelzik, E., Weinert, J., Zhao, Q., & Wolf, G. A. (2008). Factors influencing falling number in winter wheat. *European Food Research and Technology*, *226*, 1365-1371.

Waterschoot, J., Gomand, S. V., Fierens, E., & Delcour, J. A. (2015). Production, structure, physicochemical and functional properties of maize, cassava, wheat, potato, and rice starches. *Starch/Stärke*, *67*, 14-29.

Wegulo, S. N. (2012). Factors influencing deoxynivalenol accumulation in small grain cereals. *Toxins*, *4*, 1157-1180.

Yuan, J., Chung, D. S., Seib, P. A., & Wang, Y. (1998). Effect of steeping conditions on wet-milling characteristics of hard red winter wheat. *Cereal Chemistry*, *75*, 145-148.

Zhang, H., & Wang, B. (2014). Fate of deoxynivalenol and deoxynivalenol-3-glucoside during wheat milling and Chinese steamed bread processing. *Food Control*, *44*, 86-91.

5. GENERAL CONCLUSIONS

This study focused on the effectiveness of wheat wet milling technique on removing Deoxynivalenol (DON) from contaminated kernels. DON is a water-soluble *Fusarium* mycotoxin. For the proof of concept, in Chapter 3 three DON contaminated samples and a DON free control for both HRSW and DW were wet milled using the Martin process. In contrast, in Chapter 4 three wet milling processes (Martin, medium shear, and high shear) were compared for their effectiveness in removing DON. The studied samples underwent quality analysis, where the observed results suggested that DON contaminated samples suffered from a late *Fusarium* infection due to their similar quality traits with control samples and with the five-year average quality traits stated in Regional Crop Quality Reports.

All samples underwent a dry milling process in order to physically separate wheat kernels into bran, shorts, and farina (HRSW) or semolina (DW). The assessment of the DON levels in the dry milled fractions was done in Chapters 3 and 4. The obtained results showed that concentration of DON in dry milled fractions was bran>shorts>farina/semolina.

In Chapter 3 the traditional Martin wet milling method was utilized. DON was not detected in starch after wet milling farina/semolina. Similarly, DON was not detected in starch or in gluten after wet milling shorts. However, DON was detected in the gluten from HRSW (0.34-0.60 mg/kg) but not in gluten from DW. High DON levels were detected in the freeze-dried water soluble fraction in both HRSW and DW. DON was found in starch, de-starched bran, and water soluble fractions after wet milling bran. The highest concentration of DON was again found in the water soluble fractions.

With respect of Chapter 4, the obtained starch and gluten from farina/semolina wet milling did not have detectable DON levels, regardless the wet milling method. Significant differences ($P = 0.05$) among starch and gluten yield were seen between the tested wet milling methods. Overall, the traditional Martin wet milling method resulted in the highest amount of starch recovery. However, the highest amount of gluten recovery was provided by the medium shear wet milling method with DON contaminated samples (HRSW and DW).

Results from this research indicate that wet milling can be an effective method to reduce or eliminate DON from starch and gluten found in semolina/farina and shorts from HRSW and DW. The three wet milling processes were equally effective in removing DON from farina/semolina. However, they

differed in their extraction recovery of starch and gluten. The study confirmed that in fractions for human consumption DON decreased to undetectable levels after wet milling, giving some added value to contaminated grain.

6. FUTURE RESEARCH AND APPLICATION

Once proven that wet milling methods are advantageous for DON removal from the collected wheat wet milled fractions, by taking advantage of the mycotoxin's water solubility, it will be worth to investigate a series of aspects. Firstly, replicate the experiment with the same variety, having a DON infected sample and a DON free control, to fully compare the results. Then, it will be convenient to characterize the obtained wet milling fractions to assess the mycotoxin and wet milling processing impact on quality.

6.1. Same Variety Trials

The studied sets of samples in the present research were adequate for assessing the wet milling effect on the fate of DON. However, it would be very helpful to fully elucidate what the behavior of the same variety, as a DON free control and DON contaminated samples will be. Additionally, comparing several sets of varieties of both HRSW and DW will be thought-provoking so as to assess the genotype effect. In this regard, it will be convenient that the set of samples were grown at the same location so as to ensure that the environmental impact on quality relative to that of DON (FHB) is minimum. On the other hand, it will be mandatory to induce the *Fusarium* infection on one half of the tested varieties to assure DON levels, as it has been described in previous studies like in Ovando-Martinez et al. (2013). With this trial it will be possible to answer if varieties differ or have the same behavior of yielding DON free starch and gluten.

In addition, what has been described above can be complemented by assessing the environmental effect by using diverse growing locations. Additionally, the time of infection can also be controlled, in that way samples from the same wheat variety but with a severe infection and a late infection can be available, and the wet milling testing will let the genotype and infection time effect be compared.

6.2. Characterization of Wet Milled Fractions

Once proven that the extracted starch and gluten fractions have mycotoxin levels below the limit of quantification, its use for human food industry is assured. Therefore, the next stage is to assess their quality.

6.2.1. Starch Characterization

For determining starch purity and the proportion of damaged granules, the total starch and damaged starch assays will be convenient (Megazyme International, Ireland Ltd., Co.). The most common methods for characterizing starch pasting properties is by the utilization of Rapid Visco Analyzer (RVA) (Newport Scientific Pty. Ltd., Australia). Additionally, thermal properties so as to know the onset, peak, and end gelatinization temperatures will be done with Differential Scanning Calorimeter (DSC) (TA instruments, New Castle, DE). Additionally, the degree of crystallinity of a sample can be determined by X-Ray diffraction technique. The obtained results can be compared with a commercial pure starch sample, and based on the characterization results the extracted starch from mycotoxin contaminated samples by wet milling can be considered a potential functional ingredient for the food industry.

6.2.2. Gluten Characterization

The extracted gluten will undergo the determination of protein content to assess its purity (AACC International approved method 46-30.01). Then a bread making quality test to study gluten vitality will be revealed. For this purpose, the methodology described by Sayaslan, Seib, & Chung (2010) can be followed, which states that wheat flour has to be blended with the isolated gluten to reach a known protein content, so as to proceed with a bread baking straight-dough procedure (AACC International approved method 10-10.03).

6.3. References

AACC International. *Approved methods of analysis* (11th Ed.). Methods 46-30.01, 10-10.03. Available online only. St. Paul, MN: AACCI.

Ovando-Martinez, M., Ozsisli, B., Anderson, J., Whitney, K., Ohm, J.-B., & Simsek, S. (2013). Analysis of deoxynivalenol and deoxynivalenol-3-glucoside in hard red spring wheat inoculated with Fusarium graminearum. *Toxins*, *5*, 2522-2532.

Sayaslan, A., Seib, P. A., & Chung, O. K. (2010). Properties of starch and vital gluten isolated from wheat flour by three different wet-milling methods. *Journal of Applied Biological Sciences*, *4*, 51-56.

APPENDIX A. ANOVA TABLES

Table A1. Analysis of variance for HRSW samples proximate analysis.

Parameter	Sources of variation	DF	Type III SS	MS	*F* value	
1000-kernel weight	Rep	3	0.109	0.036	0.12	
	Sample	3	23.348	7.783	24.99	**
	Error	6	1.868	0.311		
Protein	Rep	3	0.095	0.0318	1.49	
	Sample	3	19.763	6.588	308.07	**
	Error	9	0.192	0.021		
Total starch	Rep	3	9.050	3.017	1.62	
	Sample	3	17.714	5.905	3.17	
	Error	9	16.768	1.863		
Ash	Rep	3	0.005	0.002	0.48	
	Sample	3	0.223	0.074	23.17	**
	Error	9	0.029	0.003		
Falling number	Rep	3	2750.917	916.972	7.59	
	Sample	3	121313.250	40437.750	334.73	**
	Error	6	724.833	120.506		

DF= Degrees of Freedom; SS= Sum of Square; MS= Mean square; and **= significant at $P \leq 0.05$.

Table A2. Analysis of variance for HRSW samples dry milling.

Parameter	Sources of variation	DF	Type III SS	MS	*F* value	
Farina fraction	Rep	3	2.556	0.852	6.64	
	Sample	3	59.310	19.770	154.12	**
	Error	9	1.155	0.128		
Shorts fraction	Rep	3	0.340	0.113	1.03	
	Sample	3	0.731	0.244	2.21	
	Error	9	0.994	0.110		
Bran fraction	Rep	3	1.075	0.358	20.21	
	Sample	3	58.589	19.530	1101.60	**
	Error	9	0.160	0.177		

DF= Degrees of Freedom; SS= Sum of Square; MS= Mean square; and **= significant at $P \leq 0.05$.

Table A3. Analysis of variance for HRSW samples DON levels in WWF and farina.

Parameter	Sources of variation	DF	Type III SS	MS	*F* value	
DON WWF	Rep	3	0.477	0.159	0.73	
	Sample	3	55.386	18.462	84.49	**
	Error	9	1.967	0.219		
DON farina	Rep	3	0.259	0.086	1.09	
	Sample	3	16.492	5.497	69.39	**
	Error	9	0.713	0.079		

DF= Degrees of Freedom; SS= Sum of Square; MS= Mean square; and **= significant at $P \leq 0.05$.

Table A4. Analysis of variance for HRSW samples washed farina starch.

Parameter	Sources of variation	DF	Type III SS	MS	*F* value	
Total starch	Rep	3	9.342	3.114	3.04	
	Sample	3	29.555	9.852	9.61	**
	Error	9	9.231	1.026		
Crude starch yield	Rep	3	10.578	3.526	3.07	
	Sample	3	1.997	0.666	0.58	
	Error	9	10.348	1.150		
Extracted starch purity	Rep	3	84.114	28.038	2.94	
	Sample	3	20.920	6.973	0.73	
	Error	9	85.841	9.538		
Net starch yield	Rep	3	256.338	85.446	2.98	
	Sample	3	48.060	16.020	0.56	
	Error	9	257.861	28.651		
Starch recovery	Rep	3	279.280	93.093	1.44	
	Sample	3	116.252	38.751	0.60	
	Error	9	581.499	64.611		
DON level in extracted starch	Rep	3	0	0	.	
	Sample	3	0	0	.	
	Error	9	0	0		

DF= Degrees of Freedom; SS= Sum of Square; MS= Mean square; and **= significant at $P \leq 0.05$.

Table A5. Analysis of variance for HRSW samples washed farina gluten.

Parameter	Sources of variation	DF	Type III SS	MS	*F* value	
Total protein	Rep	3	0.400	0.132	0.64	
	Sample	3	21.753	7.251	34.89	**
	Error	9	1.870	0.208		
Crude gluten yield	Rep	3	0.078	0.026	1.53	
	Sample	3	1.472	0.491	28.93	**
	Error	9	0.153	0.017		
Extracted gluten purity	Rep	3	0.052	0.017	0.34	
	Sample	3	69.711	23.237	456.97	**
	Error	9	0.458	0.051		
Net gluten yield	Rep	3	1.918	0.639	1.52	
	Sample	3	35.369	11.790	28.06	**
	Error	9	3.781			
Gluten recovery	Rep	3	36.505	12.168	1.00	
	Sample	3	252.173	84.058	6.94	**
	Error	9	109.067	12.119		
DON level in extracted gluten	Rep	3	0.138	0.046	1.62	
	Sample	3	0.728	0.243	8.55	**
	Error	9	0.256	0.028		

DF= Degrees of Freedom; SS= Sum of Square; MS= Mean square; and **= significant at $P \leq 0.05$.

Table A6. Analysis of variance for HRSW samples washed farina water soluble fraction.

Parameter	Sources of variation	DF	Type III SS	MS	*F* value	
Freeze-dried water solubles fraction	Rep	3	0.025	0.008	0.53	
	Sample	3	0.104	0.035	2.20	
	Error	9	0.142	0.016		
Crude water soluble fraction yield	Rep	3	0.602	0.201	0.52	
	Sample	3	2.481	0.827	2.13	
	Error	9	3.503	0.389		
DON level in water soluble fraction	Rep	3	199.290	66.430	3.96	
	Sample	3	6128.358	2042.786	121.86	**
	Error	9	150.867	16.763		

DF= Degrees of Freedom; SS= Sum of Square; MS= Mean square; and **= significant at $P \leq 0.05$.

Table A7. Analysis of variance for HRSW samples washed shorts.

Parameter	Sources of variation	DF	Type III SS	MS	*F* value	
DON level in shorts fraction	Rep	3	0.302	0.101	0.48	
	Sample	3	103.933	34.644	163.95	**
	Error	9	1.902	0.211		
Extracted starch in shorts fraction	Rep	3	0.058	0.019	0.12	
	Sample	3	1.738	0.579	3.51	
	Error	9	1.483	0.165		
Crude shorts starch yield	Rep	3	5.494	1.831	0.12	
	Sample	3	180.418	60.139	3.83	
	Error	9	141.353	15.706		
DON level in extracted shorts starch	Rep	3	0	0	.	
	Sample	3	0	0	.	
	Error	9	0	0		
Extracted gluten in shorts fraction	Rep	3	0.014	0.005	0.56	
	Sample	3	0.280	0.093	11.46	**
	Error	9	0.073	0.008		
Crude shorts gluten yield	Rep	3	1.288	0.429	0.55	
	Sample	3	26.942	8.981	11.50	**
	Error	9	7.028	0.781		
DON level in extracted shorts gluten	Rep	3	0	0	.	
	Sample	3	0	0	.	
	Error	9	0	0		
Freeze-dried water soluble in shorts	Rep	3	0.007	0.002	0.89	
	Sample	3	0.013	0.004	1.64	
	Error	9	0.024	0.003		
Crude shorts water soluble yield	Rep	3	0.684	0.228	0.87	
	Sample	3	1.284	0.428	1.64	
	Error	9	2.347	0.261		
DON level in shorts water soluble	Rep	3	46.893	15.631	0.24	
	Sample	3	15149.196	5049.732	76.29	**
	Error	9	595.740	66.193		

DF= Degrees of Freedom; SS= Sum of Square; MS= Mean square; and **= significant at $P \leq 0.05$.

Table A8. Analysis of variance for HRSW samples washed bran.

Parameter	Sources of variation	DF	Type III SS	MS	*F* value	
DON level in bran fraction	Rep	3	2.562	0.854	1.00	
	Sample	3	305.458	101.820	119.48	**
	Error	9	7.670	0.852		
Extracted starch in bran fraction	Rep	3	0.407	0.136	2.44	
	Sample	3	0.432	0.144	2.59	
	Error	9	0.499	0.055		
Crude bran starch yield	Rep	3	39.311	13.103	2.43	
	Sample	3	46.090	15.363	2.85	
	Error	9	48.539	5.393		
DON level in extracted bran starch	Rep	3	0.177	0.059	2.69	
	Sample	3	2.703	0.901	40.99	**
	Error	9	0.198	0.022		
Extracted de-starched bran fraction	Rep	3	1.701	0.567	5.99	
	Sample	3	0.825	0.280	2.91	
	Error	9	0.852	0.095		
Crude de-starched bran yield	Rep	3	162.931	54.310	5.99	
	Sample	3	73.960	24.653	2.72	
	Error	9	81.541	9.060		
DON level in de-starched bran	Rep	3	3.166	1.055	2.53	
	Sample	3	5.989	1.966	4.78	**
	Error	9	3.758	0.418		
Freeze-dried water soluble bran fraction	Rep	3	0.044	0.015	5.17	
	Sample	3	0.025	0.008	2.88	
	Error	9	0.026	0.003		
Crude bran water soluble yield	Rep	3	4.325	1.442	5.00	
	Sample	3	2.395	0.798	2.77	
	Error	9	2.597	0.289		
DON level in bran water soluble	Rep	3	551.002	183.667	0.88	
	Sample	3	8492.649	2830.883	13.52	**
	Error	9	1885.042	209.449		

DF= Degrees of Freedom; SS= Sum of Square; MS= Mean square; and **= significant at $P \leq 0.05$.

Table A9. Analysis of variance for DW samples proximate analysis.

Parameter	Sources of variation	DF	Type III SS	MS	*F* value	
1000-kernel weight	Rep	3	3.428	1.143	1.02	
	Sample	3	199.193	66.398	59.3	**
	Error	9	10.078	1.120		
Protein	Rep	3	0.081	0.027	2.43	
	Sample	3	14.639	4.880	441.66	**
	Error	9	0.099	0.011		
Total starch	Rep	3	8.947	2.982	2.34	
	Sample	3	34.437	11.479	8.99	**
	Error	9	11.490	1.277		
Ash	Rep	3	0.027	0.009	1.92	
	Sample	3	0.158	0.053	11.38	**
	Error	9	0.042	0.005		
Falling number	Rep	3	768.500	256.167	0.61	
	Sample	3	9397.500	3132.500	7.51	**
	Error	9	3756.000	417.333		

DF= Degrees of Freedom; SS= Sum of Square; MS= Mean square; and **= significant at $P \leq 0.05$.

Table A10. Analysis of variance for DW samples dry milling.

Parameter	Sources of variation	DF	Type III SS	MS	*F* value	
Semolina fraction	Rep	3	0.216	0.072	0.06	
	Sample	3	28.019	9.340	7.88	**
	Error	9	10.664	1.185		
Shorts fraction	Rep	3	0.413	0.138	0.15	
	Sample	3	11.203	3.734	4.05	**
	Error	9	8.296	0.922		
Bran fraction	Rep	3	0.155	0.052	0.66	
	Sample	3	55.353	18.451	234.50	**
	Error	9	0.708	0.079		

DF= Degrees of Freedom; SS= Sum of Square; MS= Mean square; and **= significant at $P \leq 0.05$.

Table A11. Analysis of variance for DW samples DON levels in WWF and semolina.

Parameter	Sources of variation	DF	Type III SS	MS	*F* value	
DON WWF	Rep	3	5.682	1.894	2.05	
	Sample	3	223.671	74.557	80.67	**
	Error	9	8.318	0.924		
DON semolina	Rep	3	1.155	0.385	1.35	
	Sample	3	85.141	28.380	99.58	**
	Error	9	2.565	0.285		

DF= Degrees of Freedom; SS= Sum of Square; MS= Mean square; and **= significant at $P \leq 0.05$.

Table A12. Analysis of variance for DW samples washed semolina starch.

Parameter	Sources of variation	DF	Type III SS	MS	*F* value	
Total starch	Rep	3	11.947	3.982	1.38	
	Sample	3	94.510	31.503	10.93	**
	Error	9	25.941	2.882		
Crude starch yield	Rep	3	1.055	0.352	1.43	
	Sample	3	1.157	0.386	1.56	
	Error	9	2.220	0.247		
Extracted starch purity	Rep	3	28.839	9.613	1.32	
	Sample	3	17.856	5.952	0.82	
	Error	9	65.355	7.262		
Net starch yield	Rep	3	24.776	8.259	1.36	
	Sample	3	40.655	13.552	2.23	
	Error	9	54.617	6.069		
Starch recovery	Rep	3	133.263	44.421	2.00	
	Sample	3	542.915	180.972	8.13	**
	Error	9	200.291	22.255		
DON level in extracted starch	Rep	3	0	0	.	
	Sample	3	0	0	.	
	Error	9	0	0		

DF= Degrees of Freedom; SS= Sum of Square; MS= Mean square; and **= significant at $P \leq 0.05$.

Table A13. Analysis of variance for DW samples washed semolina gluten.

Parameter	Sources of variation	DF	Type III SS	MS	*F* value	
Total protein	Rep	3	1.165	0.388	4.19	
	Sample	3	23.647	7.882	84.98	**
	Error	9	0.835	0.093		
Crude gluten yield	Rep	3	0.010	0.003	0.25	
	Sample	3	0.470	0.157	11.45	**
	Error	9	0.123	0.014		
Extracted gluten purity	Rep	3	0.144	0.048	0.49	
	Sample	3	9.048	3.016	30.56	**
	Error	9	0.888	0.099		
Net gluten yield	Rep	3	0.248	0.083	0.25	
	Sample	3	12.033	4.011	11.95	**
	Error	9	3.021	0.336		
Gluten recovery	Rep	3	38.632	12.877	0.95	
	Sample	3	62.407	20.802	1.53	
	Error	9	122.116	13.568		
DON level in extracted gluten	Rep	3	0	0	.	
	Sample	3	0	0	.	
	Error	9	0	0		

DF= Degrees of Freedom; SS= Sum of Square; MS= Mean square; and **= significant at $P \leq 0.05$.

Table A14. Analysis of variance for DW samples washed semolina water soluble fraction.

Parameter	Sources of variation	DF	Type III SS	MS	*F* value	
Freeze-dried water soluble fraction	Rep	3	0.088	0.029	10.54	
	Sample	3	0.187	0.062	22.43	**
	Error	9	0.025	0.003		
Crude water soluble fraction yield	Rep	3	2.142	0.714	10.48	
	Sample	3	4.916	1.639	24.06	**
	Error	9	0.613	0.068		
DON level in water solubles fraction	Rep	3	25.152	8.384	0.57	
	Sample	3	18860.588	6286.863	429.64	**
	Error	9	131.697	14.633		

DF= Degrees of Freedom; SS= Sum of Square; MS= Mean square; and **= significant at $P \leq 0.05$.

Table A15. Analysis of variance for DW samples washed shorts.

Parameter	Sources of variation	DF	Type III SS	MS	*F* value	
DON level in shorts fraction	Rep	3	4.447	1.482	5.13	
	Sample	3	46.986	15.662	54.19	**
	Error	9	2.601	0.289		
Extracted starch in shorts fraction	Rep	3	0.301	0.100	4.2	
	Sample	3	0.850	0.283	11.86	**
	Error	9	0.215	0.024		
Crude shorts starch yield	Rep	3	30.093	10.031	4.20	
	Sample	3	85.723	28.574	11.96	**
	Error	9	21.511	2.390		
DON level in extracted shorts starch	Rep	3	0	0	.	
	Sample	3	0	0	.	
	Error	9	0	0		
Extracted gluten in shorts fraction	Rep	3	0.002	0.001	0.29	
	Sample	3	0.243	0.081	32.54	**
	Error	9	0.022	0.002		
Crude shorts gluten yield	Rep	3	0.216	0.072	0.30	
	Sample	3	24.135	8.045	33.51	**
	Error	9	2.161	0.240		
DON level in extracted shorts gluten	Rep	3	0	0	.	
	Sample	3	0	0	.	
	Error	9	0	0		
Freeze-dried water soluble in shorts	Rep	3	0.012	0.004	0.85	
	Sample	3	0.057	0.019	4.13	**
	Error	9	0.041	0.005		
Crude shorts water soluble yield	Rep	3	1.184	0.395	0.83	
	Sample	3	5.857	1.952	4.11	**
	Error	9	4.280	0.476		
DON level in shorts water soluble	Rep	3	1096.695	365.565	1.77	
	Sample	3	6739.427	2246.476	10.85	**
	Error	9	1863.335	207.037		

DF= Degrees of Freedom; SS= Sum of Square; MS= Mean square; and **= significant at $P \leq 0.05$.

Table A16. Analysis of variance for DW samples washed bran.

Parameter	Sources of variation	DF	Type III SS	MS	*F* value	
DON level in bran fraction	Rep	3	5.877	1.959	3.91	
	Sample	3	656.448	218.816	436.42	**
	Error	9	4.513	0.501		
Extracted starch in bran fraction	Rep	3	0.099	0.033	2.24	
	Sample	3	0.013	0.004	0.28	
	Error	9	0.132	0.015		
Crude bran starch yield	Rep	3	8.980	2.993	1.81	
	Sample	3	1.413	0.471	0.29	
	Error	9	14.847	1.650		
DON level in extracted bran starch	Rep	3	0.173	0.058	5.50	
	Sample	3	8.906	2.969	283.75	**
	Error	9	0.094	0.010		
Extracted de-starched bran fraction	Rep	3	0.054	0.018	8.4	
	Sample	3	1.145	0.382	177.92	**
	Error	9	0.019	0.002		
Crude de-starched bran yield	Rep	3	5.222	1.741	7.76	
	Sample	3	111.084	37.028	165.10	**
	Error	9	2.019	0.224		
DON level in de-starched bran	Rep	3	0.030	0.010	0.44	
	Sample	3	1.545	0.515	22.88	**
	Error	9	0.203	0.023		
Freeze-dried water soluble bran fraction	Rep	3	0.041	0.014	0.83	
	Sample	3	0.459	0.153	9.21	**
	Error	9	0.149	0.017		
Crude bran water soluble yield	Rep	3	3.980	1.327	0.80	
	Sample	3	47.123	15.708	9.52	**
	Error	9	14.852	1.650		
DON level in bran water soluble	Rep	3	107.056	35.685	4.46	
	Sample	3	10647.235	3549.078	443.25	**
	Error	9	72.063	8.007		

DF= Degrees of Freedom; SS= Sum of Square; MS= Mean square; and **= significant at $P \leq 0.05$.

Table A17. Analysis of variance for HRSW samples proximate analysis.

Parameter	Sources of variation	DF	Type III SS	MS	*F* value	
1000-kernel weight	Rep	3	3.91	1.303	4.83	
	Infection	1	33.62	33.62	124.52	**
	Error	3	0.81	0.27		
Protein	Rep	3	0.008	0.003	0.12	
	Infection	1	0.125	0.125	5.66	
	Error	3	0.07	0.022		
Total starch	Rep	3	8.231	2.744	0.57	
	Infection	1	0.414	0.414	0.09	
	Error	3	14.517	4.840		
Ash	Rep	3	0.002	0.001	0.67	
	Infection	1	0.017	0.017	14.20	**
	Error	3	0.004	0.001		
Falling number	Rep	3	1981.375	660.458	1.32	
	Infection	1	23871.125	23871.125	47.67	**
	Error	3	1502.375	500.792		

DF= Degrees of Freedom; SS= Sum of Square; MS= Mean square; and **= significant at $P \leq 0.05$.

Table A18. Analysis of variance for DW samples proximate analysis.

Parameter	Sources of variation	DF	Type III SS	MS	*F* value	
1000-kernel weight	Rep	3	1.570	0.523	1.13	
	Infection	1	111.005	111.005	240.44	**
	Error	3	1.385	0.461		
Protein	Rep	3	0.038	0.013	0.38	
	Infection	1	1.577	1.577	47.80	**
	Error	3	0.099	0.033		
Total starch	Rep	3	2.662	0.887	0.29	
	Infection	1	0.111	0.111	0.04	
	Error	3	9.206	3.069		
Ash	Rep	3	0.007	0.002	0.34	
	Infection	1	0.230	0.230	32.81	**
	Error	3	0.021	0.007		
Falling number	Rep	3	2782.375	927.458	0.61	
	Infection	1	1485.125	1485.125	0.97	
	Error	3	4588.375	1529.458		

DF= Degrees of Freedom; SS= Sum of Square; MS= Mean square; and **= significant at $P \leq 0.05$.

Table A19. Analysis of variance for HRSW samples dry milling.

Parameter	Sources of variation	DF	Type III SS	MS	*F* value	
Farina fraction	Rep	3	0.223	0.075	0.19	
	Wet milling method (WM)	2	0.226	0.113	0.28	
	Infection (Inf)	1	62.514	62.514	156.41	**
	WM*Inf	2	0.051	0.025	0.06	
	Error	15	5.995	0.400		
Shorts fraction	Rep	3	0.126	0.042	0.20	
	Wet milling method (WM)	2	0.045	0.023	0.11	
	Infection (Inf)	1	5.307	5.307	25.15	**
	WM*Inf	2	0.133	0.067	0.32	
	Error	15	3.165	0.211		
Bran fraction	Rep	3	0.192	0.064	0.46	
	Wet milling method (WM)	2	0.110	0.055	0.39	
	Infection (Inf)	1	104.249	104.249	744.56	**
	WM*Inf	2	0.056	0.028	0.20	
	Error	15	2.100	0.140		

DF= Degrees of Freedom; SS= Sum of Square; MS= Mean square; and **= significant at $P \leq 0.05$.

Table A20. Analysis of variance for HRSW samples DON levels in dry milled fractions.

Parameter	Sources of variation	DF	Type III SS	MS	*F* value	
DON level in WWF	Rep	3	0.827	0.276	0.83	
	Wet milling method (WM)	2	0.007	0.003	0.01	
	Infection (Inf)	1	213.394	213.394	645.13	**
	WM*Inf	2	0.007	0.003	0.01	
	Error	15	4.962	0.331		
DON level in farina	Rep	3	0.280	0.093	1.09	
	Wet milling method (WM)	2	0.999	0.500	5.86	**
	Infection (Inf)	1	54.091	54.091	634.2	**
	WM*Inf	2	0.999	0.500	5.86	**
	Error	15	1.279	0.085		
DON level in shorts	Rep	3	5.740	1.913	1.97	
	Wet milling method (WM)	2	4.840	2.420	2.49	
	Infection (Inf)	1	347.446	347.446	357.09	**
	WM*Inf	2	4.840	2.420	2.49	
	Error	15	14.595	0.973		
DON level in bran	Rep	3	3.628	1.209	1.78	
	Wet milling method (WM)	2	0.053	0.027	0.04	
	Infection (Inf)	1	541.844	541.844	797.34	**
	WM*Inf	2	0.053	0.027	0.04	
	Error	15	10.194	0.680		

DF= Degrees of Freedom; SS= Sum of Square; MS= Mean square; and **= significant at $P \leq 0.05$.

Table A21. Analysis of variance for HRSW samples washed farina starch.

Parameter	Sources of variation	DF	Type III SS	MS	*F* value	
Total starch	Rep	3	52.885	17.628	102.27	
	Wet milling method (WM)	2	0	0	0	
	Infection (Inf)	1	0.108	0.108	0.63	
	WM*Inf	2	0	0	0	
	Error	15	2.586	0.172		
Crude starch yield	Rep	3	15.545	5.182	2.16	
	Wet milling method (WM)	2	357.010	178.505	74.45	**
	Infection (Inf)	1	1.963	1.963	0.82	
	WM*Inf	2	21.787	10.893	4.54	**
	Error	15	35.967	2.398		
Extracted starch purity	Rep	3	34.838	11.613	1.68	
	Wet milling method (WM)	2	199.400	99.700	14.4	**
	Infection (Inf)	1	0.108	0.108	0.02	
	WM*Inf	2	60.412	30.206	4.36	**
	Error	15	103.836	6.922		
Net starch yield	Rep	3	110.144	36.715	1.76	
	Wet milling method (WM)	2	7290.678	3645.339	174.55	**
	Infection (Inf)	1	22.402	22.402	1.07	
	WM*Inf	2	193.538	96.769	4.63	**
	Error	15	313.271	20.885		
Starch recovery	Rep	3	460.661	153.554	4.17	
	Wet milling method (WM)	2	12096.266	6048.133	164.12	**
	Infection (Inf)	1	28.254	28.254	0.77	
	WM*Inf	2	504.611	252.305	6.85	**
	Error	15	552.774	36.852		
DON level in extracted starch	Rep	3	0	0	.	
	Wet milling method (WM)	2	0	0	.	
	Infection (Inf)	1	0	0	.	
	WM*Inf	2	0	0	.	
	Error	15	0	0		

DF= Degrees of Freedom; SS= Sum of Square; MS= Mean square; and **= significant at $P \leq 0.05$.

Table A22. Analysis of variance for HRSW samples washed farina gluten.

Parameter	Sources of variation	DF	Type III SS	MS	*F* value	
Total protein	Rep	3	0.023	0.008	0.19	
	Wet milling method (WM)	2	0.148	0.074	1.90	
	Infection (Inf)	1	3.725	3.725	95.68	**
	WM*Inf	2	0.001	0.001	0.02	
	Error	15	0.584	0.039		
Crude gluten yield	Rep	3	1.980	0.660	2.80	
	Wet milling method (WM)	2	728.444	364.222	1543.70	**
	Infection (Inf)	1	2.231	2.231	9.46	**
	WM*Inf	2	6.172	3.086	13.08	**
	Error	15	3.539	0.236		
Extracted gluten purity	Rep	3	25.105	8.368	1.97	
	Wet milling method (WM)	2	4044.442	2022.221	476.69	**
	Infection (Inf)	1	2893.478	2893.478	682.07	**
	WM*Inf	2	1899.375	949.688	223.87	**
	Error	15	63.633	4.242		
Net gluten yield	Rep	3	8.222	2.741	3.50	
	Wet milling method (WM)	2	826.146	413.073	527.45	**
	Infection (Inf)	1	3.500	3.500	4.47	
	WM*Inf	2	15.852	7.926	10.12	**
	Error	15	11.747	0.783		
Gluten recovery	Rep	3	60.525	20.175	1.16	
	Wet milling method (WM)	2	10944.048	5472.024	315.06	**
	Infection (Inf)	1	2689.095	2689.095	154.83	**
	WM*Inf	2	1354.941	677.471	39.01	**
	Error	15	260.523	17.368		
DON level in extracted gluten	Rep	3	0	0	.	
	Wet milling method (WM)	2	0	0	.	
	Infection (Inf)	1	0	0	.	
	WM*Inf	2	0	0	.	
	Error	15	0	0		

DF= Degrees of Freedom; SS= Sum of Square; MS= Mean square; and **= significant at $P \leq 0.05$.

Table A23. Analysis of variance for HRSW samples washed farina water soluble fraction.

Parameter	Sources of variation	DF	Type III SS	MS	*F* value	
Freeze-dried water soluble fraction	Rep	3	0.153	0.051	1.17	
	Wet milling method (WM)	2	9.982	4.991	114.54	**
	Infection (Inf)	1	0.004	0.004	0.09	
	WM*Inf	2	0.006	0.003	0.06	
	Error	15	0.654	0.044		
Crude water soluble fraction yield	Rep	3	0.700	0.233	2.22	
	Wet milling method (WM)	2	1.703	0.851	8.08	**
	Infection (Inf)	1	0.013	0.013	0.12	
	WM*Inf	2	0.024	0.012	0.11	
	Error	15	1.580	0.105		
DON level in water soluble fraction	Rep	3	1.210	0.403	0.02	
	Wet milling method (WM)	2	294.483	147.241	7.33	**
	Infection (Inf)	1	31464.535	31464.53	1566.1	**
	WM*Inf	2	311.998	155.999	7.77	**
	Error	15	301.348	20.090		

DF= Degrees of Freedom; SS= Sum of Square; MS= Mean square; and **= significant at $P \leq 0.05$.

Table A24. Analysis of variance for DW samples dry milling.

Parameter	Sources of variation	DF	Type III SS	MS	*F* value	
Semolina fraction	Rep	3	6.363	2.121	3.05	
	Wet milling method (WM)	2	4.071	2.035	2.93	
	Infection (Inf)	1	37.985	37.985	54.65	**
	WM*Inf	2	0.774	0.387	0.56	
	Error	15	10.425	0.695		
Shorts fraction	Rep	3	6.242	2.081	3.05	
	Wet milling method (WM)	2	0.876	0.438	0.64	
	Infection (Inf)	1	32.341	32.341	47.42	**
	WM*Inf	2	0.535	0.267	0.39	
	Error	15	10.230	0.6820		
Bran fraction	Rep	3	0.296	0.099	0.42	
	Wet milling method (WM)	2	1.590	0.795	3.42	
	Infection (Inf)	1	0.227	0.227	0.98	
	WM*Inf	2	0.849	0.424	1.83	
	Error	15	3.483	0.232		

DF= Degrees of Freedom; SS= Sum of Square; MS= Mean square; and **= significant at $P \leq 0.05$.

Table A25. Analysis of variance for DW samples DON levels in dry milled fractions.

Parameter	Sources of variation	DF	Type III SS	MS	*F* value	
DON level in WWF	Rep	3	5.043	1.681	1.78	
	Wet milling method (WM)	2	0.794	0.397	0.42	
	Infection (Inf)	1	852.513	852.513	902.01	**
	WM*Inf	2	0.794	0.397	0.42	
	Error	15	14.177	0.945		
DON level in farina	Rep	3	0.835	0.278	0.79	
	Wet milling method (WM)	2	0.779	0.389	1.11	
	Infection (Inf)	1	456.936	456.936	1300.6	**
	WM*Inf	2	0.497	0.249	0.71	
	Error	15	5.270	0.351		
DON level in shorts	Rep	3	3.757	1.252	2.23	
	Wet milling method (WM)	2	0.369	0.184	0.33	
	Infection (Inf)	1	361.176	361.176	644.3	**
	WM*Inf	2	0.369	0.184	0.33	
	Error	15	8.409	0.561		
DON level in bran	Rep	3	2.617	0.872	0.86	
	Wet milling method (WM)	2	11.712	5.856	5.77	**
	Infection (Inf)	1	1829.361	1829.361	1802.8	**
	WM*Inf	2	11.712	5.856	5.77	**
	Error	15	15.221	1.015		

DF= Degrees of Freedom; SS= Sum of Square; MS= Mean square; and **= significant at $P \leq 0.05$.

Table A26. Analysis of variance for DW samples washed semolina starch.

Parameter	Sources of variation	DF	Type III SS	MS	*F* value	
Total starch	Rep	3	84.592	28.197	16.21	
	Wet milling method (WM)	2	0	0	0	
	Infection (Inf)	1	8.298	8.298	4.77	**
	WM*Inf	2	0	0	0	
	Error	15	26.091	1.739		
Crude starch yield	Rep	3	2.389	0.796	1.22	
	Wet milling method (WM)	2	1042.875	521.437	801.48	**
	Infection (Inf)	1	55.916	55.916	85.95	**
	WM*Inf	2	29.248	14.624	22.48	**
	Error	15	9.759	0.651		
Extracted starch purity	Rep	3	6.375	2.125	0.20	
	Wet milling method (WM)	2	71.839	35.920	3.39	
	Infection (Inf)	1	0.005	0.005	0	
	WM*Inf	2	8.462	4.231	0.40	
	Error	15	158.810	10.587		
Net starch yield	Rep	3	18.863	6.288	1.62	
	Wet milling method (WM)	2	9253.103	4623.552	1195.58	**
	Infection (Inf)	1	249.880	248.880	64.57	**
	WM*Inf	2	175.118	87.559	22.63	**
	Error	15	58.046	3.870		
Starch recovery	Rep	3	132.837	44.279	2.62	
	Wet milling method (WM)	2	16423.906	8211.953	485.80	**
	Infection (Inf)	1	326.348	326.348	19.31	**
	WM*Inf	2	307.497	153.748	9.10	**
	Error	15	253.559	16.904		
DON level in extracted starch	Rep	3	0	0	.	
	Wet milling method (WM)	2	0	0	.	
	Infection (Inf)	1	0	0	.	
	WM*Inf	2	0	0	.	
	Error	15	0	0		

DF= Degrees of Freedom; SS= Sum of Square; MS= Mean square; and **= significant at $P \leq 0.05$.

Table A27. Analysis of variance for DW samples washed semolina gluten.

Parameter	Sources of variation	DF	Type III SS	MS	*F* value	
Total protein	Rep	3	0.137	0.046	0.83	
	Wet milling method (WM)	2	0.176	0.088	1.59	
	Infection (Inf)	1	12.136	12.136	220.09	**
	WM*Inf	2	0.093	0.047	0.85	
	Error	15	0.827	0.055		
Crude gluten yield	Rep	3	2.413	0.804	1.06	
	Wet milling method (WM)	2	718.257	359.128	475.39	**
	Infection (Inf)	1	6.639	6.639	8.79	**
	WM*Inf	2	7.520	3.760	4.98	**
	Error	15	11.332	0.755		
Extracted gluten purity	Rep	3	119.694	39.898	4.57	
	Wet milling method (WM)	2	2671.802	1335.901	153.02	**
	Infection (Inf)	1	1991.402	1991.402	228.10	**
	WM*Inf	2	1038.320	519.160	59.47	**
	Error	15	130.954	8.730		
Net gluten yield	Rep	3	4.442	1.481	0.66	
	Wet milling method (WM)	2	1219.851	609.926	272.48	**
	Infection (Inf)	1	27.204	27.204	12.15	**
	WM*Inf	2	13.139	6.570	2.93	
	Error	15	33.576	2.238		
Gluten recovery	Rep	3	200.677	66.892	0.90	
	Wet milling method (WM)	2	24366.418	12183.209	164.75	**
	Infection (Inf)	1	763.991	763.991	10.33	**
	WM*Inf	2	1335.441	667.720	9.03	**
	Error	15	1109.241	73.949		
DON level in extracted gluten	Rep	3	0	0	.	
	Wet milling method (WM)	2	0	0	.	
	Infection (Inf)	1	0	0	.	
	WM*Inf	2	0	0	.	
	Error	15	0	0		

DF= Degrees of Freedom; SS= Sum of Square; MS= Mean square; and **= significant at $P \leq 0.05$.

Table A28. Analysis of variance for DW samples washed semolina water soluble fraction.

Parameter	Sources of variation	DF	Type III SS	MS	*F* value	
Freeze-dried water soluble fraction	Rep	3	0.141	0.047	0.92	
	Wet milling method (WM)	2	11.961	5.981	117.25	**
	Infection (Inf)	1	0.010	0.010	0.20	
	WM*Inf	2	0.187	0.093	1.83	
	Error	15	0.765	0.051		
Crude water soluble fraction yield	Rep	3	0.824	0.275	1.83	
	Wet milling method (WM)	2	6.009	3.004	19.97	**
	Infection (Inf)	1	0.027	0.027	0.18	
	WM*Inf	2	1.211	0.606	4.03	**
	Error	15	2.256	0.150		
DON level in water soluble fraction	Rep	3	4.359	1.453	0.68	
	Wet milling method (WM)	2	22.696	11.484	5.36	**
	Infection (Inf)	1	48210.506	48210.50	22495.6	**
	WM*Inf	2	37.658	18.829	8.79	**
	Error	15	32.147	2.143		

DF= Degrees of Freedom; SS= Sum of Square; MS= Mean square; and **= significant at $P \leq 0.05$.

APPENDIX B. FIGURES

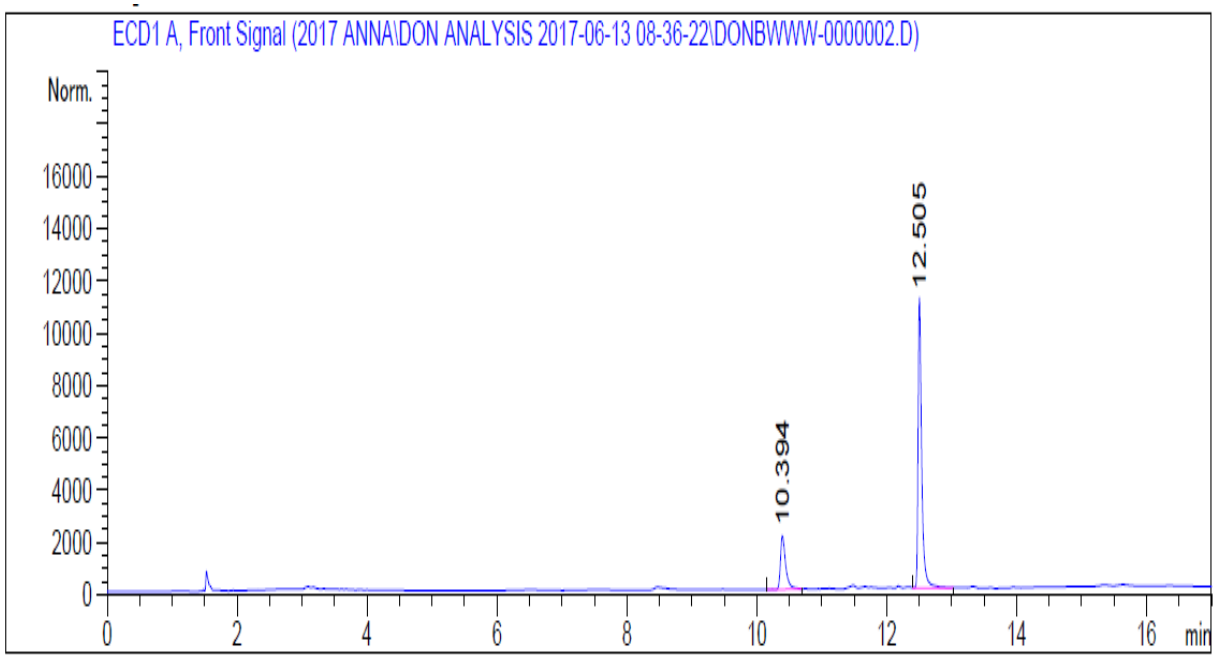

Figure B1. Example of GC-ECD chromatogram.
From left to right, the first peak represents the presence of DON, and the second peak represents the internal standard.