PRE-HARVEST GLYPHOSATE USE DURING WHEAT CULTIVATION: EFFECTS ON WHEAT CHEMISTRY AND HUMAN GUT MICROBIOTA

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Glyphosate is the most widely used herbicide in the world which is sometimes utilized as a pre-harvest desiccant during wheat cultivation. It inhibits the 5-enolpyruvylshikimate-3-phosphate synthase enzyme in the shikimic acid pathway in plants. Although this pathway is not found in humans, it is present in human gut microbiota. In this context, the goal of this study was to examine the effect of pre-harvest glyphosate application on wheat quality, biochemical characteristics and human gut microbiota. The results of this study indicated that the effects of glyphosate on wheat quality is more pronounced when applied at soft dough stage. Glyphosate lowered B-type starch granules and vice versa for A-type granules and it changed the proportions of rapidly digestible and slowly digestible starch. Starch amylopectin chain length distribution was also impacted although the effects were different when applied at the two stages. Glyphosate lowered the molecular weight of SDS extractable and unextractable proteins when applied as a desiccant. Additionally, shikimic acid accumulation was especially high in samples treated at soft dough stage. As for gut microbiota, the results indicated that glyphosate may not have a profound impact on metabolite production by gut microbiota, although there maybe effects on bacterial population dynamics. Overall, the current study indicates that glyphosate applied pre-harvest has some effects on wheat physicochemical properties and gut microbiota. In the context of wheat chemistry, the effects of glyphosate on the shikimic acid pathway, followed by subsequent accumulation of shikimic acid and effects on carbon flow may cause changes in the biosynthesis of starch and proteins. Glyphosate could impact enzyme activity, as it can interact with metals that are required as co-factors in enzyme catalyzed reactions. Glyphosate’s effect on intermolecular interactions between starch and protein, and other macromolecules such as dietary fiber, may also influence the overall chemistry of plant components. Although the effects of
glyphosate on gut microbiota are not clear-cut, this exploratory study is a stepping stone in this area of research. In conclusion, the observations made in this study should be investigated further to determine causal links and relationships.
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DEDICATION

To my beloved grandmother Mrs. Rita Pathinather
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GENERAL INTRODUCTION

Glyphosate is the most widely used herbicide in the world. It is a non-selective, broad spectrum, post-emergence herbicide, therefore controls a wide range of different species (Powles 2008). In the case of wheat, glyphosate can be used prior to planning, pre-emergence, pre-harvest and post-harvest (EPA, 2016). Although glyphosate is effective in weed control, side effects of this herbicide on the crop itself, micro and macro organisms and plant diseases have been reported (Dorn et al. 2013). As such, in the context of environmental issues, use of glyphosate has been a compelling issue during the last few years.

In a study conducted to determine the effect of pre-harvest application of glyphosate on grain quality, it was found that glyphosate at a level of 1.0 kg/ha decreased the germination energy and the length and weight of primary roots, and when applied at 2 kg/ha, glyphosate decreased the thousand kernel weight (Jaskulski and Jaskulska 2014). The study also found that wheat desiccation using this herbicide limits the emergence and weight of seedlings. Moreover, glyphosate drifts to non-target crops, results in growth aberrations and reductions in yield (Kutman et al. 2013). Deeds et al. (2006) studied the effect of glyphosate drift on winter wheat yield and injury, where they found that injury and yield reductions increased with increasing concentration of this herbicide, and that the effects were greater when glyphosate was applied at the jointing stage. Rolder et al. (2007) also made similar observations, where reductions in yields were observed in wheat as a result of glyphosate drift. Yield reductions were indicated by reductions in spike density, spikelets per spike and seed weight.

In addition to yield loss, glyphosate also causes many negative side effects. Baur et al. (1977) studied the effect of glyphosate on sorghum and wheat in relation to growth and development. Glyphosate was found to decrease the fresh weight of seedlings in this study and...
inhibit the production of basal buds at the optimum temperature. Buds were produced at temperatures above the optimum in this experiment. Genotoxic effects of glyphosate were reported by Nardemir et al. (2015), where they found that this herbicide caused DNA alterations and methylations. DNA methylation is an important step involved in biological events affecting DNA integrity and function, and the main function of methylation in eukaryotes is related to protecting genome integrity. Additionally, the genomic stability index decreased with increasing concentrations of glyphosate indicating that higher concentrations are more effective in giving rise to DNA alterations.

Glyphosate has also been implicated as a contributing factor with reference to many human diseases. Samsel and Seneff (2013a) argue that glyphosate inhibits the P450 enzymes and are thus toxic to mammals. This class of enzymes have detoxifying effects on xenobiotics, thus, when inactive, the damaging effects of food borne residual chemicals are enhanced. In humans, majority of the glyphosate ingested (about 98%) is eliminated from the body; however, the 2% that remains is metabolized to aminomethylphosphonic acid, a major breakdown product (Williams et al. 2000). Additionally, according to a recent review, glyphosate interrupts the shikimic acid pathway in the gut microbiome, leading to increased incidence of celiac disease (Samsel and Seneff 2013b). Glyphosate has also been implicated in causing dysbiosis in the gut microbiome of agriculturally important animals like poultry and cattle (Shehata et al. 2013 and Kruger et al. 2013). Thus, it is important to assess if there are any interactions between glyphosate and human gut microbiota.

In this context, the aims of this study were to determine the effect of glyphosate timing on hard red spring wheat cultivars grown in North Dakota. Effects on end-use quality and the fine chemistry of wheat starch and proteins were studied. A field study was used to determine the
effects of glyphosate timing on wheat quality, and the rheological behavior of starch and proteins, while a greenhouse experiment was used to determine the effect of glyphosate timing on the fine chemistry of starch and proteins. Additionally, interactions between glyphosate and human gut microbiota were evaluated using an *in vitro* fecal fermentation study.

In this context, the objectives of this study were as follows,

I. Analyze the effect of glyphosate on wheat quality characteristics ranging from kernel quality to baking quality

II. Determine the effect of glyphosate on the physicochemical properties of wheat starch and protein composition

III. Evaluate the effect of glyphosate on protein deposition in wheat by analyzing changes in the fine chemistry of wheat proteins.

IV. Determine the effect of glyphosate on starch deposition by analyzing the fine chemistry of wheat starch polymers.

V. Determine if there is an interaction between glyphosate and gut microbiota by conducting an *in vitro* fecal fermentation study.
CHAPTER 1. LITERATURE REVIEW

In 1974, when glyphosate was first introduced as a herbicide, 0.36 million kilograms of the chemical was used by farmers in the United States, and in 1995, the amount used increased to 12.5 million kilograms (Benbrook 2016). With the introduction of glyphosate resistant crops, the use of glyphosate increased dramatically, reaching 36 million kilograms by the year 2000. The same trend is seen globally, where the use of glyphosate increased from 51 million kilograms to 747 million kilograms from 1996 to 2014. As demonstrated by these statistics, glyphosate has now become the most widely used herbicide in the world. The driving forces for the dramatic increase in the use of glyphosate in the United States and worldwide are the commercialization of glyphosate resistant crops, new application methods and new agricultural practices, such as the use of glyphosate as a pre-harvest desiccant. Glyphosate is used as a harvest-aid in crops, such as wheat, barley and edible beans. As mentioned earlier, some studies have reported negative effects of glyphosate on plant physiological characteristics (Dorn et al. 2013), and there is evidence that this herbicide interacts with gut microbiota (Shehata et al. 2013).

1.1. Glyphosate: chemistry and translocation through the plant

1.1.1. Mode of action of glyphosate

Glyphosate is referred to as 2-(phosphonomethylamino) acetic acid according to IUPAC definitions. It is also known as N-(phosphonomethyl)-glycine or glyphosate. Its molecular formula is C₃H₈NO₅P.
Glyphosate acts as an herbicide by inhibiting the enzyme enolpyruvylshikimate phosphate synthase (EPSPS) in the shikimic acid pathway, thereby interfering in the production of essential amino acids such as, phenylalanine, tyrosine and tryptophan (Williams et al. 2000). One hypothesis is that glyphosate is safe for species in the animal kingdom because this pathway is not found in the animal kingdom. Also, it has been reported that the active site for EPSPS in higher plants is very highly conserved (Cajacob et al. 2004). Additionally, glyphosate has been found to be most effective when it comes into direct contact with foliage, and through subsequent translocation throughout the plant.

Figure 1.2. Shikimic acid pathway in plants. Reprinted from Williams et al. (2000)
The EPSPS enzyme catalyzes the reaction, during which the enolpyruvyl moiety of phosphoenopyruvate is transferred to shikimate-3-phosphate (Dill 2005). This is a critical step in the shikimic acid pathway and is crucial for the synthesis of aromatic amino acids, hormones, and other plant metabolites, such as flavonoids, lignins and phenolic compounds. One of the substrates for the EPSPS enzyme is phosphoenolpyruvate, as shown in Figure 1.2, and glyphosate is a transition state analog for this compound (Duke and Powles 2008). Inhibition of EPSPS by glyphosate causes reduced feedback inhibition of the shikimic acid pathway, and leads to increased carbon flow to shikimate-3phosphate, which later converts to shikimate. The actual mechanism which kills glyphosate treated plants is still unclear. The assumption of many researchers is that plant death is caused by insufficient aromatic amino acids, however, there is evidence that increased carbon flow to shikimate, caused by the deregulation of the pathway could deprived carbon for other essential biochemical pathways leading to plant death.

Franz et al. (1997) reported that binding site for glyphosate on the EPSPS enzyme overlaps closely with the binding site for phosphoenolpyruvate. Additionally, glyphosate has a competitive mechanism with respect to the binding of phosphoenolpyruvate to EPSPS, however, it is said to have an uncompetitive mechanism with respect to binding to shikimate-3-phosate, which leads to the formation of glyphosate:EPSPS:shikimate-3phosphate complexes, which are very stable. Therefore, interactions with glyphosate essentially shuts down the shikimic acid pathway. Sikorski and Gruys (1997) reviewed the chemical mode of action of glyphosate and reported that glyphosate forms a stable ternary complex with EPSPS and shikimate-3phosphate, and that this complex causes the herbicidal effects of glyphosate similar to the observations by Franz et al. (1997). Additionally, some studies suggest that glyphosate acts as a transition state analog for phosphoenolpyruvate.
1.1.2. Glyphosate uptake and translocation

Kirkwood et al. (2000) reported that glyphosate is mainly taken up by plant surfaces, and that the uptake rate of leaves varies among different plant species. Additionally, it was reported that glyphosate uses diffusion to travel across the plant cuticle. Afterwards, it uses phloem tissues to translocate from the leaf tissue to metabolic active tissues that act as sucrose sinks (Siehl 1997). Thus, toxic levels of glyphosate reach actively growing tissue or organs of affected plants. For example, meristems, young roots and leaves and storage organs can be affected. As explained above, good uptake, effective translocation mechanisms through the plant to actively growing sites, nil or limited degradation once translocated, as well as slow mode of action make glyphosate a highly effective herbicide. Although glyphosate translocates rapidly through the phloem, Sikorski and Gruys (1997) reported that plant death could take several days or sometimes weeks. However, Mike et al. (1983) reported that biochemical effects of glyphosate can be observed within hours of application. As for degradation of glyphosate, Rueppel et al. (1977) found that it is degraded by microorganisms in the soil, which ultimately produce ammonia, inorganic phosphate and CO$_2$.

1.2. Glyphosate: history, use and resistance

1.2.1. Historical perspective

Glyphosate or N-phosphonomethyl-glycine was first synthesized in 1950 by Henri Martin at a pharmaceutical company called Cilag (Franz et al. 1997). However, pharmaceutical applications of the glyphosate are not reported in literature. In 1959, Johnson and Johnson acquired Cilag along with its research samples, including glyphosate, which were sold to the chemical company Aldrich Chemical (Dill et al. 2010). Although Aldrich Chemical investigated potential applications glyphosate, they did not report any biological activity. During this period,
at Monsanto – Inorganic Division, many aminomethylphosphonic acid compounds were being tested as potential water-softening agents. These compounds were also tested for herbicidal effects, with only two compounds showing such effects. However, their activity was too low to be considered for further research as herbicides. During this time, Dr. John Franz of Monsanto began further testing of these substances, which lead to the synthesis of glyphosate in May of 1970, which was tested in a greenhouse study. The positive results of glyphosate’s herbicidal effects quickly gained attention and after further research, Monsanto commercialized glyphosate and introduced it as Roundup®. The herbicidal effects of the chemical was first described by in scientific literature by Baird et al. (1971) and glyphosate was patented by Monsanto as a herbicide in the US patent 3,799,758 (U.S. Patent no. 3,799,758).

1.2.2. Glyphosate use in agriculture

As a broad-spectrum post-emergence herbicide glyphosate can control the growth of a wide range of plant species, examples include, annual grasses, annual broadleaves, johnson grass, quackgrass, yellow nutsedge, cool season pasture and turf grasses, cattail, Canada thistle, hemp dogbane, Jerusalem artichoke, poison ivy, and multiflora rose. According to Benbrook (2016) glyphosate is mostly applied after harvest to prevent weed infestation of winter crops (pre-planting) or sometimes after sowing before the emergence of new crop plants (post planting –pre emergence). It is also used before harvest (pre-harvest) as a weed control and to accelerate the maturation of crops (desiccation), especially in the case of small grain crops. The phytotoxic effects of glyphosate are exemplified by visual observations like chlorosis, which is usually followed by necrosis, morphological leaf deformations and damage to roots and rhizomes (Szekacs and Darvas 2012). Additionally, Haderlie et al. (1978) reported that glyphosate accumulates in the meristematic tissue of affected plants.
Since the expiration of Monsanto’ patent for glyphosate, other agrochemical companies have also introduced glyphosate containing herbicides (Dill et al. 2010). Such products are usually water solutions of glyphosate salts, with most products also containing specific surfactants. Examples for such products include, Touchdown® (Syngenta, Basel, Switzerland), GlyphoMAX® (Dow AgroSciences LLC, Indianapolis, IN) and Gly Star® (Albaugh, Inc., Ankeny, IA). The National Pesticide Information Retrieval System records show that approximately 50 different agricultural products with glyphosate are in the commercial market. Such products contain glyphosate salts, with different cation(s), the molecular mass of the salts differ among products. Thus, for comparison, the active ingredients concentration is reported as acid equivalent or glyphosate equivalent, which refers to the free acid form of glyphosate (Szekacs and Darvas 2012). Various non-ionic surfactants are used in glyphosate formulations to facilitate the uptake of glyphosate by the plant (Riechers et al. 1995). In the case of glyphosate, polyethyloxylated tallowamine (POEA) is the most commonly used surfactant (Szekacs and Darvas 2012).

When using glyphosate on cereal grains and crops, such as wheat and barley, the EPA registration lists the types of application as application types as those recommended for annual and perennial crops (pre-planting, at planting, post-emergence and post-harvest), wiper applicator (feed barley and wheat only) and pre-harvest (feed barley and wheat only) (EPA, 2016). When using as a pre-harvest aid during wheat cultivation, it is recommended to be applied when the grain has 30% or less moisture and it is recommended to have 7 days between application and harvest.
1.2.3. **Statistics of glyphosate use in the U.S. and worldwide**

As mentioned earlier, glyphosate use in the United States increased from 0.36 million kg in 1974 to 12.5 million kg in 1995 (Benbrook 2016). In 1995, glyphosate was the seventh most widely used herbicide in the United States. Atrazine and metolachlor, both used during corn cultivation, were at the top of the list, with soil fumigants used mostly on fruits and vegetables, metam-sodium, methylbromide, dichloropropene came in third – fifth. At this time, most farmers relied on one broad-leaf herbicide, 2, 4-D, which was the sixth most widely used herbicide. With the introduction of glyphosate resistant crops around 1995, glyphosate use rapidly increased to 36 million kg by 2000, and by 2010 1.37 billion kg of glyphosate was used. Given these statistics, glyphosate use in the agriculture sector increased about 300-fold between 1974 and 2014. When considering glyphosate usage for specific crops, soybeans, corn and cotton account for approximately 80% of glyphosate used in the United States. On a worldwide scale, glyphosate use increased more than 12-fold considering agricultural and non-agricultural usage, with 67 million kg used in 1995 to 826 million used in 2014. In the last decade alone, 6.1 billion kg of glyphosate has been applied, which accounts for approximately 72% of total use between 1974 and 2014.

1.2.4. **Glyphosate resistance crops and glyphosate resistant weeds**

Glyphosate resistant crops, also known by the trade name Roundup Ready®, are not affected by the herbicidal action of glyphosate because they contain an EPSPS enzyme that is insensitive to glyphosate’s mode of action (Dill 2005). The majority of glyphosate resistant crops have the bacterial EPSPS (also known as CP₄) that was isolated from *Agrobacterium* sp., which is insensitive to glyphosate. It is suggested that binding of glyphosate to this enzyme system is prevented by conformation changes caused by changes in amino acid sequences. In 1996,
glyphosate resistant soybean was introduced to the commercial market in the United States, and since then, glyphosate resistant crops have been used by farmers at a very rapid pace. Today, there are six major glyphosate resistance crops: soybean, corn, cotton, canola, alfalfa and sugar beet (Green 2018), and approximately 56% was glyphosate used worldwide is used on glyphosate resistant crops (Benbrook 2016).

Since the introduction of glyphosate in the early 1970s, it was extremely effective as an herbicide, which resulted in farmers being heavily reliant on this chemical (Green 2018). This unprecedented use of glyphosate and decline in the use of other herbicides, which was fueled by glyphosate resistant crops resulted in selection pressure amongst weeds, which eventually lead to the formation of glyphosate resistant weeds (Powles 2008). Sammons and Gaines (2014) reported that glyphosate resistance mechanisms in glyphosate resistant weeds can be categorized as, (i) target-site resistance which affects the interaction between the herbicide and the target enzyme, (ii) metabolism, which means causing a chemical modification to the herbicide, (iii) exclusion of the herbicide from the target using structural barriers or physiologically active transporters, and (iv) avoidance where the weed species can avoid the toxic result caused by the herbicide. Examples of glyphosate resistant weeds include, *Amaranthus palmeri, A. tuberculatus, Ambrosia artemisiifolia, A trifida, Lolium rigidum* and *Sorghum halepense* (Green 2018).

1.3. Wheat growth and physiology

To understand how external factors affect biomacromolecule deposition in wheat or wheat quality, it is important to understand how wheat development progresses until physiological maturation. Environmental factors as well as agrochemical usage could affect wheat yield and other quality parameters differently depending on the stage of physiological
maturation during which crops come into contact with such stresses (Savin and Slafer 1991; Yan and Hunt 2001).

1.3.1. Developmental stages of wheat

Crop development can be described as “a sequence of phenological events controlled by external factors, determining changes in the morphology and/or function of some organs” (Miralles and Slafer 1999). In terms of crop development, it is a continuity of vegetative, reproductive and grain filling stages, and when passing through these phases the crop grows organs and completes its life cycle. During this time, interactions between genetic and environmental factors affect the number of primordia initiated. To describe the stages of wheat development, several scales are currently being used, where some scales describe morphological changes (Gardner et al. 1985), whereas others focus on growth stages visible without dissection of the shoot apex (Zadoks et al. 1974). Figure 1.3 below shows the developmental stages of wheat.

Figure 1.3. Stages of wheat development. Reprinted with permission from Guo et al. (2015)
As shown in Figure 1.3, there are three major stages of wheat development, namely vegetative, reproductive and grain filling (Miralles and Slafer 1999). Wheat is in vegetative state upon sowing (Sw) and emergence (Em), until the initiation of the first double ridge (DR). During the reproductive phase, events such as terminal spikelet initiation and heading (Hd) take place. Once anthesis (At) begins, wheat enters the grain filling phase, with beginning of grain filling (BGF) starting soon after flowering. A short time after reaching physiological maturity (PM), wheat is ready to be harvested (Hv). During the reproductive phase, factors such as spikelets per spike, grains per spike and spike survival rate affect wheat yield, and when wheat reach the grain filling stage, weight per grain becomes a determining factor for yield. When describing wheat development stages, in general the Feekes scale or Zadoks scales are used. Wheat kernel development can also be classified into five stages according to the morphological changes in the wheat kernel as well as the moisture content of the grain. Figure 1.4. shows this categorization.

![Figure 1.4. Stages of wheat kernel development: watery (a) milk stage (b), soft dough (c), hard dough (d) and ripe (e). Reprinted with permission from Simmons et al. (1985)](image)

Each of the kernel developmental stages can be described using the Feekes scale or the Zadoks scale as mentioned earlier. Table 1.1. summarizes this information. As kernels reach physiological maturity the moisture content decreases (Herbek and Lee 2009). For example, at milk stage the moisture content is quite high, and at soft dough stage, when kernels become essentially dry, the moisture level decreases to approximately 40%. At hard dough stage, the
moisture is approximately 30%. Wheat harvest is recommended to be performed at approximately 13-15% moisture to avoid any post-harvest spoilage or damage. Pre-harvest desiccants, such as glyphosate are recommended to be sprayed at ripe stage to quicken the process of decreasing the moisture content of grains in order to facilitate uniform ripening and timely harvest. It is vital that grain is harvested in a timely manner because further ripening could reduce kernel test weight (Herbek and Lee 2009).

Table 1.1. Kernel development stages

<table>
<thead>
<tr>
<th>Stage</th>
<th>Description</th>
<th>Feekes scale</th>
<th>Zadoks scale</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk development</td>
<td>Kernel watery ripe</td>
<td>10.54</td>
<td>71</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Late milk</td>
<td>11.1</td>
<td>77</td>
<td>Milky ripe, noticeable increase in solids</td>
</tr>
<tr>
<td>Dough development</td>
<td>Early dough</td>
<td>11.1</td>
<td>83</td>
<td>Mealy ripe, kernels soft but dry</td>
</tr>
<tr>
<td></td>
<td>Soft dough</td>
<td>11.2</td>
<td>85</td>
<td></td>
</tr>
<tr>
<td>Ripening</td>
<td>Hard dough</td>
<td>11.2</td>
<td>87</td>
<td>Physiological maturity</td>
</tr>
<tr>
<td></td>
<td>Kernel hard, can be split</td>
<td>11.3</td>
<td>91</td>
<td>Ripe for harvest, straw dead</td>
</tr>
<tr>
<td></td>
<td>using thumb nail</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Kernel hard, cannot</td>
<td>11.4</td>
<td>92</td>
<td></td>
</tr>
<tr>
<td></td>
<td>be split by thumbnail</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Source: Herbek and Lee (2009)

1.3.2. Chemistry of wheat starch

The main storage carbohydrate of plants is starch (Copeland et al. 2009). It is stored as insoluble, semi-crystalline granules in storage tissues such as, grains, tubers and roots. As for the composition of starch, two types of polymers are involved, namely amylose and amylopectin (Delcour and Hoseney 2010b). Amylose is linear and consists of α-D-glucose united linked via α-(1-4) bonds. The molecular weight of amylose ranges from 80,000 to 1,000,000, and the weight varies among amongst different plant species, as well as within species. The main factor
influencing molecular weight of amylose is grain maturity. Similar to amylose, amylopectin is also composed of α-(1-4) linked α-D glucose units. Unlike amylose, amylopectin is a branched structure with α-(1-6) linkages. Amylopectin is a very large molecule, which could have a molecular weight of $10^8$. Amylopectin polymeric structures can be categorized as A chains, which do not carry any branches, therefore contain only α-(1-4), linkages, B chains contain both α-(1-4) and α-(1-6) linkages, and C chains contain both types of linkages as well as a reducing end. The variability, in terms of chain length and molecular weight is due to the complex process of starch biosynthesis (Copeland et al. 2009). The moisture content of starch granules has been reported as approximately 10%, and amylose and amylopectin account for 98-99% of the dry weight of native starch granules, whereas the remainder is lipids, minerals and phosphorous.

Starch gelatinization is the basis for many types of food products, and processes like bread baking are dependent on the optimum gelatinization of starch, which results in desirable texture (Olkku and Rha 1978). Bechtel et al. (1964) described wheat starch gelatinization in two stages. During the first step, a small amount of starch granules swell between 60-70°C, allowing the disruption of accessible amorphous sites. During the second step, rapid swelling occurs between 80-90°C, allowing the disruption of more accessible sites. Upon continues heating, it has been observed that swollen granules disrupt into fragments. Gelatinization begins at the hilum of the granule and subsequently moves to the peripheral regions (Singh et al. 2003). During the swelling process, granules swell several times its original size and rupture, leading to amylose leaching, which forms a network structure. According to Horstmann et al. (2017), starch functionality in food systems is affected by factors such as, granule size and shape, starch crystallinity, amylose-amylopectin ratio, packing density, lipids, encapsulation of granules, swelling power and solubility, gelatinization, retrogradation (re-association of starch polymers
into a packed structure during the subsequent cooling phase after heating starch) and rheological behavior.

### 1.3.3. Starch deposition in wheat

In wheat, starch is deposited as granules in structures called amyloplasts, and each of these structures contain one granule (Delcour and Hoseney 2010b). Wheat granules are lenticular and round, and 20-35 µm and 2-10 µm in size, respectively. In wheat, approximately 25% of starch is amylose and 75% is amylopectin. Wheat starch granules have different size ranges because granule growth initiates at different times during grain filling (BeMiller 2007). A-type granules are larger and lenticular in shape, with a diameter of more than 10 µm, while B-type granules are smaller and spherical with 1-5 (≤ 10) µm in diameter. A-type granules and B-type granules form approximately 1-10% and 90-99% of granules by number. However, by weight A-types granules take up between 50 and 87% by starch weight.

Wheat starch and other carbohydrate compounds are produced using CO$_2$ fixed during the grain filling process (Jenner et al. 1991). The rate of starch deposition in wheat is affected by the balance between the ability of the plant to produce the substrate (source-limited) and the capability of the grain to use it (sink-limited). However, Fischer et al. (1977) reported that in most healthy plants, starch deposition is mainly limited by factors associated with sink-limiting. Therefore, grain related factors determine the rate of starch deposition in wheat.

Wheat development takes a sigmoidal shape (Stone and Savin 1999). That is, at the beginning, individual kernel mass increases rapidly, followed by a short lag phase, until physiological maturation, after which an almost stationery phase is achieved. Physiological maturity is reached approximately 50 days after anthesis, and rapid water loss occurs after reaching maturation. During the lag phase, starch synthesis sites become active, and A-type amyloplasts are activated.
first (Bechtel et al. 1990). Thus, A-type starch granules are produced during the period of 4-12 days after anthesis. Each amyloplast gives rise to one granule during this production phase. Although A-type starch granules are produced first, such granules reach their maximum size until later during the grain filling process. B-type amyloplasts are activated approximately 15 days after anthesis, and the production of B-type starch granules continue until physiological maturity.

Several enzymes are involved in the starch biosynthesis process such as, ADP pyrophosphorylase, which produces ADP-glucose (glucose donor in the synthesis process) from hexose-phosphates, soluble and granule bound starch synthase which catalyzes the α(1→4) bond formation during synthesis, starch branching enzymes which catalyze the formation of α(1→6) branch points and debranching enzymes which trim newly synthesized amylopectin chains, allowing the formation of crystalline structures (Copeland et al. 2009). Such enzymes involved in starch biosynthesis can be found in multiple isoforms, and their activity is impacted by factors such as, temporal and spatial differences in expression at genetic and enzyme levels, natural diversity of enzymes and environmental conditions.

1.3.4. Chemistry of wheat proteins

Wheat proteins are found in the endosperm of the kernel, and are termed gluten forming proteins (Bonomi et al. 2013). These proteins form a continuous matrix around starch granules. Gluten proteins are responsible for the viscoelastic properties of wheat dough, which are very important in baking performance. As Shewry (2009) highlighted, the key characteristic of wheat is the ability to form dough, and gluten forming protein found in the endosperm are responsible for imparting this property. From a geneticist’s perspective, the annotation of gluten proteins is complex due to extensive polymorphism (Shewry et al. 1986). Additionally, genes responsible
for gluten proteins have resulted from duplication and translocation events, which resulted in insertions and deletions of peptide sequences and amino acid substitutions. The amino acid composition of wheat proteins has been reported previously and glutamine and proline appear to be the most abundant amino acids found in wheat, primarily due to the abundance of these amino acids in gluten proteins (Shewry 2009). Table 1.2 below shows the distribution of amino acids in wheat.

Table 1.2. Distribution of amino acids in wheat proteins

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Soluble proteins</th>
<th>Gluten proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wheat</td>
<td>Flour</td>
</tr>
<tr>
<td>Asp</td>
<td>4.7</td>
<td>3.7</td>
</tr>
<tr>
<td>Thr</td>
<td>2.4</td>
<td>2.4</td>
</tr>
<tr>
<td>Ser</td>
<td>4.2</td>
<td>4.4</td>
</tr>
<tr>
<td>Glu</td>
<td>30.3</td>
<td>34.7</td>
</tr>
<tr>
<td>Pro</td>
<td>10.1</td>
<td>11.8</td>
</tr>
<tr>
<td>Gly</td>
<td>3.8</td>
<td>3.4</td>
</tr>
<tr>
<td>Ala</td>
<td>3.1</td>
<td>2.6</td>
</tr>
<tr>
<td>Val</td>
<td>3.6</td>
<td>3.4</td>
</tr>
<tr>
<td>Met</td>
<td>1.2</td>
<td>1.3</td>
</tr>
<tr>
<td>Cys</td>
<td>2.8</td>
<td>2.8</td>
</tr>
<tr>
<td>Ile</td>
<td>3.0</td>
<td>3.1</td>
</tr>
<tr>
<td>Leu</td>
<td>6.3</td>
<td>6.6</td>
</tr>
<tr>
<td>Tyr</td>
<td>2.7</td>
<td>2.8</td>
</tr>
<tr>
<td>Phe</td>
<td>4.6</td>
<td>4.8</td>
</tr>
<tr>
<td>His</td>
<td>2.0</td>
<td>1.9</td>
</tr>
<tr>
<td>Lys</td>
<td>2.3</td>
<td>1.9</td>
</tr>
<tr>
<td>Arg</td>
<td>4.0</td>
<td>3.1</td>
</tr>
<tr>
<td>Trp</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>NH₃</td>
<td>3.5</td>
<td>3.9</td>
</tr>
</tbody>
</table>

Source: Shewry et al. (2009)

Wheat gluten proteins are composed of glutenin and gliadin proteins. Gliadins are monomeric, whereas glutenins are polymeric with aggregates held together by disulfide bonds. Gliadins provide cohesive or viscous properties, while glutenins provide resistance to extension or elasticity. Cereal proteins are categorized into four groups according to their solubility
Albumin proteins are soluble in water, while globulins are soluble in dilute salt solutions. These two proteins are found in the germ. Gliadins and glutenins are soluble in 70% ethanol and dilute acid or base respectively and are storage proteins found in the endosperm. The molecular weight of glutenin proteins range from 500,000 to more than 10 million (Wieser 2007), whereas for gliadins it ranges from 28,000 to 50,000 (Bonomi et al. 2013).

Gliadin proteins are classified as α-, β-, γ- and ω- according to their mobility on low pH electrophoresis (Wieser 2007). However, α- and β- gliadins are frequently noted as α/β due to similarities in their amino acid sequences and electrophoretic mobility. The quantity and distribution of gliadin protein fractions has been found to be impacted by genotype and environment. However, Wieser and Kieffer (2001) determined that majority of gliadin proteins belong to either α/β- or γ- gliadins, whereas ω-gliadins is a minor constituent. The role of gliadin proteins in general and individual fractions of gliadin proteins in dough mixing characteristics is highly debated. As Barak et al. (2015) reported, gliadin proteins function as a plasticizer during dough mixing, imparting viscous flow properties and extensibility to wheat dough.

Glutenin proteins can be found as ‘glutenin macropolymers’ and such glutenin polymers have shown the greatest contribution towards favorable dough properties (Wieser 2007). Glutenin proteins can also be found as high molecular weight (HMW) or low molecular weight (LMW) molecules. The former represents 5-10 of total gluten content, while the latter represents approximately 20%. Interestingly, the amino acid composition of LMW glutenin proteins has been found to be similar to that of α/β- and γ- gliadin proteins. Barak et al. (2014) studied the role of gluten protein in dough mixing and found that glutenin proteins improve dough mixing characteristics, while gliadins have the opposite effect.
1.3.5. **Protein deposition in wheat**

Proteins are deposited in discrete membrane bound bodies during grain filling, within the developing endosperm, where starch synthesis also occurs (Stone and Savin 1999). Ribosomes are the starting point of protein production, and the synthesized proteins move though a secretory (through the endoplasmic reticulum and the Golgi apparatus) pathway to be stored in vacuoles. Similar to the shape of grain development, protein accumulation in wheat kernels is also sigmoidal. Protein involved in metabolic reactions, such as albumins and globulins are produced first during protein deposition. In fact, during the first ten days of grain development, these proteins account for approximately 90% of the total protein in the grain. However, this percentage decreases during the progression of development, and at physiological maturity, albumin and globulin proteins account for 20-30% of total protein in the grain, whereas the remainder is storage proteins. Gliadin protein synthesis initiates approximately 5 - 10 days after anthesis, and at physiological maturity, these proteins account for 30-40% of the total proteins in the kernel. Glutenin proteins are found at detectable levels approximately 20 days after anthesis, and at maturity constitute for 30-40% of the total protein. However, SDS soluble and insoluble glutenin proteins accumulate at different times during development, where SDS soluble proteins are deposited first and SDS insoluble glutenin proteins are deposited later. As such, at maturity 90% of glutenin proteins belong to the SDS soluble category. On average, the molecular mass of proteins increases as grain progress through the grain filling phase, as glutenin proteins are deposited later. Additionally, the gliadin to glutenin ratio decreases with time.

1.3.6. **Bread wheat quality**

Bread wheat quality parameters range from kernel quality characteristics, such as thousand kernel weight and test weight to baking quality characteristics such as loaf volume,
crumb grain and texture and crumb color. Many researchers have associated wheat proteins as a major factor governing wheat quality traits. Starch provides the structural framework during bread development and is also important source of energy. Damaged starch has been shown to affect bread quality parameters, such as loaf volume and crumb texture (Bird 1957). The structural properties of starch also affect the thermal behavior of wheat starch as characterized by differential scanning calorimetry (Delcour and Hoseney 2010b). Additionally, starch swelling capacity is important during gelatinization and retrogradation. Branlard and Dardevet (1985), Ng and Bushuk (1988) and Gupta et al. (1992) found that HMW-glutenin subunits are high associated with favorable dough properties like dough strength and extensibility. The role of gliadin proteins in bread systems is still unclear, with researchers presenting different views on the different types of gliadin proteins. Uthayakumaran et al. (1999) and Macritchie (1987) found that addition of gliadin proteins to base flour has negative effects on dough mixing properties. Contrary to this view, Huebner and Bietz (1986) and Wrigley et al. (1981) determined that specific gliadin proteins may have a favorable effect on end-product quality. Additionally, Khatkar et al. (1995) and Uthayakumaran et al. (2000) determined that a fine balance between glutenin and gliadin proteins is important in dough and baking quality, where gliadin proteins contribute flow properties or viscous nature to wheat dough, whereas glutenin proteins are important in providing elasticity.

1.4. **Effects of glyphosate on plant physiology, protein and starch chemistry, and yield**

As explained earlier, the effects of glyphosate on plant physiology has been extensively studied. Glyphosate is broken down to aminomethylphosphonic acid (AMPA) by microorganisms once it reaches the soil (Franz et al. 1997), and a similar mechanism of breakdown has been proposed for plants by Reddy et al. (2004). As such, glyphosate and its
breakdown products can be present in plant tissue as a result of glyphosate degradation and/or uptake from the environment. Once glyphosate penetrates the internal plant tissue, it travels to active sites such as, roots and meristems via the vascular tissue (Satchivi et al. 2000). In a similar manner, AMPA is also translocated throughout the plant (Reddy et al. 2004). In this context, nodules, root tips and shoot apices, act as ‘sinks’ for glyphosate and AMPA. Glyphosate, which is a metal chelator, could also affect enzyme co-factors (Gomes et al. 2014). Also, AMPA has been identified as a phytotoxin, could compete with the amino acid Glycine and affect biological pathways.

Fedtke and Duke (2005) found that glyphosate reduces photosynthesis in plants by inhibiting the synthesis of carotenoids, chlorophylls, fatty acids and amino acids. By inactivating the shikimic acid pathway, the biosynthesis of secondary metabolites, such as quinones is reduced, leading to decreased photosynthesis. Zobiole et al. (2012) conducted field studies using glyphosate-resistant soybean and found that increased levels of glyphosate, and application in later growth stages impact photosynthesis, nutrient accumulation and nodulation. Additionally, glyphosate has been implicated in decreasing the amount of chlorophyll in leaves via inhibition of its biosynthesis or inducing the degradation of these molecules (Zobiole et al. 2011). This could be because glyphosate decreases the amount of Magnesium in leaves through its role as a metal chelator (Cakmak et al. 2009). From a different perspective, Serra et al. (2013) demonstrated that plants treated with AMPA have reduced levels of amino acids such as, glycine, serine and glutamate, and that this would lead to lower amount of δ-aminolevulinic acid, which is a component of the chlorophyll biosynthetic pathway.

Glyphosate also affects carbon metabolism in plants by reducing the C exchange and stomatal conductance (Zobiole et al. 2011). Additionally, Servaites et al. (1987) who studied the
effect of glyphosate on sugar beet determined that the herbicide reduced the levels of ribulose-1, 5-biphosphate (RuBP) and 3-phosphoglyceric acid, and the enzyme ribulose-1,5-biphosphate carboxylase, which together affects the rate of C fixing. Another hypothesis is that the C metabolism is affected as a result of the impact on the shikimic acid pathway. Servaites et al. (1987) also reported that glyphosate reduced starch synthesis, although it did not have an effect on sucrose synthesis and translocation. Reduced starch synthesis was observed just 4 hours after the foliar application of glyphosate. Since glyphosate impacts photosynthesis in multiple ways, and starch biosynthesis process may also be impacted, leading to changes in the starch chemistry of plants treated with this herbicide. As for possible changes in the protein chemistry of plants treated with glyphosate, the effect on the shikimic acid pathway maybe responsible.

Application of glyphosate on crops such as wheat results in severe injury to plants. Such plants can be exposed to glyphosate through drift as well as through accidental application. Fernandez et al (1994) studied the effects of glyphosate on carbon balance, transpiration, and biomass partitioning in wheat, and found that glyphosate treatment caused damage in the root system and inhibited carbon uptake and transpiration. These conditions were linked to stomatal closure; however, it is unknown if this phenomenon is a direct result of glyphosate application. Yenish and Young (2000) examined the effect of glyphosate on seed and seeding quality of soft white spring wheat. Glyphosate was applied pre-harvest, at three different stages: milk, soft dough, hard dough, seven days following the treatment at hard dough stage and 1 day prior to harvest. They found that the yield was reduced only when glyphosate was applied at the milk stage, and that the reduction ranged from 20-70% depending on the year, variety and amount of glyphosate applied. Additionally, kernel weight and germination were also reduced when the
herbicide was applied at the milk stage. Thus, they concluded that the effects of glyphosate of seed and seedling quality is dependent of the maturity stage of the crop at the time of application.

1.5. **Gut microbiota**

The gastrointestinal (GI) tract represents about 250-400 m², and is one of the largest interfaces between the host, environmental factors and antigens in the human body (Thursby and Juge 2017). The gut microbiota can be defined as the collection of bacteria, archaea and eukarya colonizing the GI tract. The gut microbiota have evolved over thousands of years together with the host to form a mutually beneficial relationship with the host (Backhed et al. 2005). Studies have found that approximately $10^{14}$ bacterial cells inhabit the GI tract. That is, the number of bacterial cells is almost ten times the amount of human cells. However, more recent findings show that the ratio between bacterial and human cells could be about 1:1 (Sender et al. 2016).

1.5.1. **Introduction to human gut microbiota**

The Metagenomics of the Human Intestinal Tract (MetaHit) and Human Microbiome Project have played a pivotal role in identifying human-associated microbial populations up to date (Hugon et al. 2015; Li et al. 2014). These projects identified 2172 different bacterial species in the human GI tract, and classified the identified species to 12 phyla, of which 93.5% belong to Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes. Three of the 12 phyla contained only one bacterial species identified in humans. For example, *Akkermansia muciniphila* was identified as the only species belonging to the phyla Verrucomicrobia phylum. Approximately 18% of the identified species were determined as strictly anaerobic, which means that they are found in the mucosal regions, such as the oral cavity and GI tract. Additionally, it has been determined that the gut microbiota composition is influenced by environmental factors, such as diet, and possibly host genetics. It is believed that the gut microbiota develops from birth.
(Thursby and Juge 2017). The GI tract is rapidly colonized after birth, and events such as illness, antibiotic use, and changes in diet cause changes in the gut bacteria population (Rodriguez et al. 2015).

According to Tremaroli and Backhed (2012) the Firmucutes phylum contains several genera including *Ruminococcus, Clostridium, Lactobacillus, Eubacterium, Faecalibacterium* and *Roseburia*, and the Bacteroidetes phylum contains complex glycan degrading genera such as *Prevotella* and *Xylanibacter*. Examples for Actinobacteria include *Collinsella* and *Bifidobacterium* and examples for Proteobacteria include, *Escherichia* and *Desulfovibrio*.

Figure 1.5 shows the distribution of different bacterial species in the GI tract, which depends on pH conditions and the requirements of the individual species.

![Figure 1.5. Distribution of normal gut microflora. Reprinted with permission from Jandhyala et al. (2015)](image-url)
1.5.2. Functions of gut microbiota and role in maintaining health

As mentioned earlier, gut microbiota have a symbiotic relationship with the host, and perform a wide range of functions, such as metabolic, immunological and protective functions (Jandhyala et al. 2015). Gut bacteria derive energy from host dietary components, especially from dietary carbohydrates. Fermentation of such carbohydrate compounds produce short chain fatty acids, such as butyrate, acetate and propionate, which act as energy sources for the host. Examples of species that ferment carbohydrate compounds include, *Bacteroides, Roseburia, Bifidobacterium, Fecalibacterium*, and *Enterobacteria*. Enzymes such as glycosyl transferases, glycoside hydrolases and polysaccharide lyases participate in such carbohydrate metabolizing reactions.

Functions of the gut microbiota include, nutrient metabolism, xenobiotic and drug metabolism, antimicrobial protection, immunomodulation and keeping the integrity of the gut barrier and structure of the GI tract (Jandhyala et al. 2015). As mentioned earlier, gut bacteria use different enzymes for the fermentation of carbohydrate compounds to produce different fatty acids. In addition to carbohydrates, gut bacteria can also metabolize proteins using microbial proteinases and peptidases. Examples include the conversion between L-histidine to histamine which used the bacterial histamine decarboxylase enzyme (Thomas et al. 2012). Other function of gut microbiota, such as synthesizing vitamin K and several compounds of vitamin B have also been reported (Albert et al. 1980; Conly and Stein 1992; Hill 1997).

The ability of gut microbiota to metabolize xenobiotics was first identified about 40 years and, recent studies by Clayton et al. (2009) showed that p-cresol, which is a microbial metabolite, can reduce the ability of the liver to metabolize acetaminophen by competitively inhibiting hepatic sulfotransferases. However, microbial β-glucoronidase interacts with the
anticancer drug irinotecan, which ultimately leads to outcomes like diarrhea, inflammation and anorexia (Wallace et al. 2010). For the purpose of antimicrobial protection, a two-tiered mucus system is employed (predominantly in the large intestine), which prevents contact between luminal microbes and epithelial cells (Johansson et al. 2008). In the small intestine, antimicrobial proteins, such as cathelicidins, C-type lectins, and (pro) defensins play a role in preventing the growth of pathogenic organisms (Hooper 2009). Additionally, immunoglobulins are also used for antimicrobial purposes (He et al. 2007).

The gut microbiota works with the innate and the adaptive immune systems to promote immunomodulation, where the gut associated lymphoid tissues, effector and regulatory T cells, IgA producing B cells, Group 3 innate lymphoid cells and resident macrophages and dendritic cells in the lamina propria participate in specific response pathways (Jandhyala et al. 2015). Several compounds produced by specific members of the gut microbiota contribute towards preserving the integrity of the gut barrier and the structure of the GI tract. For example, *Bacteroides thetaiotaomicron* is involved in the production of a small proline rich protein, which is essential for the function of desmosomes at the epithelial villus (Lutgendorff et al. 2008), and the role of microbial cell wall peptidoglycans in signaling the Toll-like receptor 2 (TLR2) mediated maintenance of tight junctions (Cario et al. 2007).

The short chain fatty acids produced during the microbial metabolic reactions/fermentation of carbohydrate compounds/dietary fiber by gut microbiota play significant role in maintaining human health (Macfarlane and Macfarlane 2012). Bacterial genera such as, *Bacteroides, Bifidobacteria, Clostridia, and Eubacteria* produce predominately produce acetate, whereas *Bacteroides, Clostridia, Propionibacteria, and Veillonella* produce propionate. *Roseburia, Feacalibacteria, Clostridia* and *Fusobactia* produce butyrate. Short chain fatty acids
can be ranked as butyrate, propionate and acetate in terms of their metabolic significance. Butyrate has been shown to participate in regulating cell growth, differentiation, cytoskeleton organization and modulation of gene expression. Additionally, butyrate is also important in preventing inflammation, inducing apoptosis of human colonic carcinoma cells, preventing the damaging effects on DNA caused by oxidative H₂O₂, inducing the expression on tight junction proteins and inducing satiety by producing butyrate from oligofructose. Propionate and acetate also participate in maintaining the immune system, preventing carcinogenesis, improving colonic function, cholesterol metabolism and inducing satiety.

In addition to the above functions, gut microbiota is also important in maintaining normal gut functions, which includes the functions explained in section 1.4.2. (Zhang et al. 2015). Gut bacteria also transforms natural compounds such as lignin to their bioactive forms (enterolignins), which provide protection against cardiovascular disease, hyperliperdemia, breast cancer, colon cancer, prostate cancer, osteoporosis and menopausal syndrome. Moreover, gut microflora play an important role in metabolizing isoflavones, which have been found to protect against various forms of cancer and osteoporosis (Atkinson et al. 2005).

1.6.  Effects of glyphosate on human health and gut microbiota

Although glyphosate is one of the most widely used chemicals in the world, some studies have illustrated the impact of it in terms of being a contributing factor to health issues.

In a review by Samsel and Seneff (2013a), an array of such issues is highlighted. Glyphosate acts by disrupting the shikimic acid pathway in plants as discussed earlier. Since this pathway is absent in humans, glyphosate is thought to be safe. However, the shikimic acid pathway is present in gut bacteria, which have an integrated relationship with the human host (Littman and Pamer 2011). As such, the health and wellbeing of the gut microbiota is very
important as it leads to the homeostasis of the immune system by helping in digestion, detoxification and synthesis of vitamins as explained earlier. Additionally, imbalances in the gut have been linked to autism spectrum disorder further highlighting the importance of the healthy and functional gut microbiome (Williams et al. 2011). In addition, celiac disease has been associated with reduced levels of various gut microorganisms such as, Enterococcus, Bifidobacteria and Lactobacillus (Sanz et al. 2011).

Shehata et al. (2013) studied the effects of glyphosate in poultry and found that this herbicide disrupts the balance in the gut microbiota, leading to the increases levels of pathogenic bacteria, and lower the levels of commensal bacteria. Additionally, Ackermann et al. (2015) studied the effect of glyphosate on microbiota and expression of botulinum neurotoxin during in vitro ruminal fermentation and found that glyphosate has inhibitory effects on some ruminal microbiota species, but caused an increase in pathogenic species, which lead them believe that glyphosate causes dysbiosis and favors the production of botulinum toxins. Similarly, Kittle et al. (2018) studied the effect on glyphosate on gastrointestinal microflora of Hawaiian green turtles, since this herbicide is frequently sprayed along the Hawaiian shorelines. Their results showed that exposure to glyphosate at levels of more than $1.76 \times 10^{-6}$ g/L caused reduced growth in bacterial isolates such as Pantoea, Proteus, Shigella and Staphylococcus, which could lead to negative effects on the overall digestion and gut health of turtles. During the last decade, an increase of Clostridium botulinum was seen in cattle in Germany, and in this context Kruger et al. (2013) studied interactions between glyphosate and gut microbiota to determine if there is any association. They found that glyphosate suppresses antagonistic effects of Enterococcus spp. on Clostridium botulinum, which could be a factor contributing towards the rise in Clostridium botulinum associated diseases in cattle. Moreover, Nielsen et al. (2018) conducted an animal
study, which showed that glyphosate has limited short-term effects on beneficial gut microbiota, because the availability of aromatic amino acids in the intestinal tract compensates for what is lost due to the inhibition of the shikimic acid pathway.

Moreover, some studies identify the interference of the enzyme cytochrome P450 by residual glyphosate as a factor that contributes to unfavorable outcomes in different organs such as the liver. Gasnier et al. (2009) found that an amount of 10 ppm of glyphosate disrupts the activity of the aromatase enzyme, in HepG2 cells, which are used the study xenobiotic toxicity. On a different note, retinoic acid plays a key role in embryonic development, and its amount is tightly regulated to facilitate optimum development (Aulehla and Pourquie 2010). When reports of birth defects in children born in regions where glyphosate based chemicals are widely used came to light, Paganelli et al. (2010) investigated the effect of low glyphosate levels on the development of frog embryos and chick embryos. The embryos treated with glyphosate showed high levels of abnormalities, and lead to the development of tadpoles with cranial deformities. The increased level of retinoic acid was suggested as the reason for the abnormalities, as an antagonist for retinoic acid prevented the abnormalities. One explanation is the suppression of a cytochrome P450 enzyme by glyphosate as demonstrated by Ray et al. (1997).

Adding to the list of health concerns caused by glyphosate, impairment of the homeostasis of serotonin is also important as it regulates mood, appetite and sleep, and also depression (Cowen 2008). Liver disease is also important in this context, as glyphosate has been linked to inducing cytokines. Tumor necrosis factor-α, which is a type if cytokine is a key factor in such diseases, as these substances can induce inflammation in the liver, by inhibiting the insulin signaling and overloading the liver with lipid compounds (Peraldi et al. 1996).
Additionally, increased exposure to glyphosate has been implicated in substantially higher risk in developing multiple myeloma (De Roos et al. 2005).

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CHAPTER 2. PRE-HARVEST GLYPHOSATE TIMING AND EFFECTS ON SPRING WHEAT QUALITY CHARACTERISTICS

2.1. Abstract

Glyphosate is the most widely used herbicide in the world. It is a non-selective, broad spectrum, post-emergence herbicide, therefore controls a wide range of different weed species. Although glyphosate is effective in weed control, side effects of this herbicide on the crop itself, micro and macro organisms and plant diseases have been reported. In this context, the objective of this study was to determine the effect of glyphosate timing on spring wheat quality characteristics, ranging from kernel quality to baking quality. For this purpose, two wheat cultivars were grown in three locations, and glyphosate was applied at the recommended rate at soft dough stage and ripe stage. The control samples were not sprayed with glyphosate. Upon harvest, kernel quality, flour quality, dough quality and baking quality were assessed using standard AACC1 methods. When glyphosate was applied at soft dough stage, kernel weight, wheat protein and wet gluten decreased significantly \((P \leq 0.05)\), in comparison to the control and treatment at ripe stage; however, gluten index significantly increased \((P \leq 0.05)\). The treatment did not show any effect on flour ash content. As for dough quality, farinograph stability, and quality number were significantly \((P \leq 0.05)\) higher when glyphosate was applied at soft dough stage, and absorption and mixing tolerance index were lower. As for baking quality, loaf volume and mix time were significantly \((P \leq 0.05)\) higher in the treated samples in comparison to the untreated, and other baking quality characteristics did not show any significant difference. Overall, the results indicate that spring wheat quality characteristics were impacted to the greatest degree when glyphosate is applied at the soft dough stage, and that glyphosate may
cause changes to the biochemical properties of wheat starch and proteins leading to significant effects on end-product quality.

2.2. Introduction

Glyphosate inhibits the 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS) enzyme found in the shikimic acid pathway (Duke and Powles 2008). The inhibition of EPSPS leads to reduced feedback in the inhibition pathway, which results in the accumulation of shikimate-3-phosphate, which is converted to shikimate or shikimic acid. Glyphosate is considered as a non-selective, broad spectrum herbicide because the EPSPS enzyme in higher plants is inhibited by glyphosate. Many researchers assume that deficiency of aromatic amino acid production in glyphosate treated plants leads to plant destruction (Williams et al. 2000). However, there are indications that the deregulation of the shikimic acid pathway is the cause for plant mortality, where increased carbon flow to shikimate-3-phosphate causes shortages in carbon required for other essential pathways (Siehl 1997). Studies conducted on sugarbeet demonstrates that the deregulation of the shikimic acid pathway results in reduced rate of ribulose bisphosphate carboxylase regeneration, photosynthesis and starch synthesis (Servaites et al. 1987).

Glyphosate is used as pre-harvest desiccant during wheat cultivation and is sprayed prior to harvest. It is also used as a harvest-aid for dry beans, lentils and soybean. Reasons such as untimely rainfall or unfavorable damp conditions makes wheat more susceptible to pre-harvest sprouting and mildew development, which makes it important to decrease the moisture content of wheat kernels close to harvest (Dexter 1998), thus herbicides such as glyphosate are used as pre-harvest desiccants to facilitate timely harvest of crops (Manthey et al. 2004). When glyphosate is used for wheat desiccation, it should be applied at kernel moisture content of 30% or less (Darwent et al. 1994b). Other herbicides such as 2,4-D and metsulfuron are also used for
pre-harvest weed control during wheat cultivation. Glyphosate causes a sudden stop in the physiological maturation process of wheat and leads to increases in the shikimic acid content as reported by Bresnahan et al. (2003). Additionally, there are indications that glyphosate application could cause the accumulation of water-soluble fiber components such as fructans in wheat kernels, as opposed to migrating towards other parts of the plant (Pollock and Cairns 1991).

Glyphosate could terminate the physiological maturation process of wheat disrupting the grain filling process, which results in negative effects on grain yield (Darwent et al. 1994b). Such effects have been observed especially when glyphosate is applied at high grain moisture levels (50% moisture content). Decreases in grain yield, test weight, kernel weight and kernel size were observed in such instances. Yenish and Young (2000) studied the effect of applying glyphosate at milk stage, soft dough stage, hard dough stage, seven days after hard dough stage and a day before harvest and determined that application at milk stage causes detrimental effects on yield. This is a clear indication that application time of glyphosate-based herbicides is critical in preserving grain yield as well as other quality parameters. Glyphosate levels of less than 1 kg ha$^{-1}$ caused detrimental effects of emergence as well as weight of seedling, at levels of 1 kg ha$^{-1}$ caused decreases in germination energy and at 2 kg ha$^{-1}$ thousand kernel weight was decreased according to a study by Jaskulski and Jaskulska (2014). Additionally, Krenchinski et al. (2017) studied the effects of several herbicides used for desiccation and found that glyphosate, paraquat, glyfosinate-ammonium, diquat and clethodium caused decreased in grain yield, while herbicides such as paraquat and clethodium caused reductions in seedling length and vigor respectively. McNeal et al. (1973) studied eight desiccants and found desiccant application does not have a profound effect on the end-product quality of hard red spring wheat. However, applying
desiccants at higher moisture levels showed negative effects on wheat protein content, loaf volume and bread texture scores. Additionally, glyphosate application caused wheat dough to become tougher requiring comparative more energy for optimal dough development (Darwent et al. 1994a). However, as in the previous study, quality parameters such as protein content and mixograph peak height, peak width and total energy of the curve were not affected by glyphosate application.

Baker’s yeast, *Saccharomyces cerevisiae* could be impacted to different degrees according to the herbicide that is used as demonstrated by Sharma et al. (2005), who studied different agrochemicals, such as endosulfan, hexaconazole, propiconazole, malathion, chlorpyriphos and deltamethrin. The presence of such chemicals suppressed yeast growth at times up to approximately 45%, which could lead to negative effects on the end-product quality of fermented goods. Low et al. (2005) investigated interactions between yeast and effects of bread leavening and reported that yeast plays a role in metabolizing glyphosate during fermentation, where approximately 21% of glyphosate is degraded within the first hour of fermentation. Similarly, Hack et al. (1997) reported that baker’s years metabolizes the herbicide atrazine. Braconi et al. (2006) also observed that glyphosate could have negative impacts during wine fermentation due to interactions with yeast. They also noted that these effects are enhanced by the different surfactants used together with glyphosate. However, Roisch and Lingens (1980) indicated that glyphosate does not affect the synthesis of aromatic amino acids in baker’s yeast at levels of 2 mmol l⁻¹.

In this context, the objective of this study was to determine the effect of glyphosate applied at different stages of maturity, namely at soft dough stage (45% moisture content) and ripe stage (30% moisture content), on different spring wheat quality characteristics ranging from
kernel quality to end-product baking performance. For this purpose, a field study was conducted where glyphosate was applied at the recommended rate, and upon harvest quality characteristics were determined according to standard AACC-I methods.

2.3. Materials and methods

2.3.1. Study design

In this study, a 3 x 3 x 2 factorial design (location x treatment x cultivar) was used with a split-plot layout, with location as the main plot, and treatment x cultivar as the sub-plot. Two wheat cultivars, Glenn and Prosper, were grown at three locations, namely, Minot, Carrington and Prosper in North Dakota. Glyphosate was sprayed at the recommended rate (1.1 kg active ingredient/ha) at soft dough stage and ripe stage, and harvested upon complete maturation. Ripe stage is considered as physiologically mature grain. A control where glyphosate was not sprayed was also used. Each treatment was repeated three times, bringing the total number of samples to 54.

2.3.2. Quality analysis of harvested samples

To determine the nitrogen content of wheat kernels, the American Association of Cereal Chemists – International (AACC-I) method 39-10.10 was used. A Dumas nitrogen analyzer was used for this purpose. The protein content was determined at 14% moisture basis (m.b). Test weight was determined using the AACC-I method 55-10.01. A sample of 10 g of cleaned wheat was used to determine thousand kernel weight using an electronic seed counter (Seedburo Equipment Count-A-Pak Model 77 Totalizer). Kernel vitreousness was determined by way of visual observations and approximate percentage of kernels having vitreous endosperm was determined. Falling number was determined according to the AACC-I method 56-81.01, and the results were reported in seconds at 14% moisture basis (m.b.)
The samples were cleaned and milled in house in the Wheat Quality laboratory facilities. A Bühler ML-202 laboratory scale mill was used for milling, and straight grade flour was blended and reported as flour extraction. The standard AACC-I method mentioned above for protein analysis was used to determine flour protein content, and AACC-I method 08-01.01 was used to measure flour ash content. To determine gluten index and wet gluten AACC-I approved method 38-12.02 was used.

Flour mixing characteristics were evaluated using farinograph analysis (Farinograph-E, C. W. Brabender) according to the AACC-I approved method 54-21.02, and the Brabender software was used to determine farinograph characteristics such as, peak time, stability, mixing tolerance, quality number and absorption. Baking tests were used to assess the breadmaking quality of the samples, for which 100 g pup loaves were used. The baking tests were performed according to the AACC-I approved method 10-09.01 with some modifications. The fermentation time was shortened to 2 hours, fungal amylase was used instead of malt power, instant dry yeast was used in place of compressed yeast and 1 mL of 10% ammonium phosphate was used in each loaf. Baking quality parameters such as loaf volume was assessed according to the AACC-I method 10-05.01 and other parameters such as, crust color, crumb color, crumb grain and texture and loaf symmetry were determined using the AACC-I method 10-12.01.

2.3.3. Statistical analysis

The SAS software (Version 9.3, SAS Institute, Cary, NC) was used to perform Analysis of Variance (ANOVA) and mean separation (Fisher’s protected LSD).
2.4. Results and discussion

2.4.1. Effects of glyphosate on kernel quality, milling quality and flour quality

In this study, quality characteristics such as 1000-kernel weight, falling number, wheat protein content, percent milling extraction, wet gluten and gluten index were significantly affected by treatment of glyphosate at soft dough stage and ripe stage.

Several quality traits also showed significant differences for cultivar x treatment and cultivar x location interactions. The cultivar x treatment interaction was significantly different for kernel vitreousness, 1000-kernel weight, falling number, wheat protein content, flour protein content, percent extraction, wet gluten, gluten index and flour ash. The location x treatment interaction was significant 1000-kernel weight, percent extraction, wet gluten and gluten index, and interaction between all three factors was significant different for percent extraction and gluten index. This data is summarized in Table 2.1.

According to Table 2.1, 1000-kernel weight was significantly ($P \leq 0.05$) lower in soft dough compared to the control and the ripe treatment indicating the early application of glyphosate can have an impact on seed weight. During the physiological maturation of wheat, the grain filling process takes place from anthesis to harvest (Stone and Savin 1999). When glyphosate is applied at soft dough, it is possible that the gain filling process is interrupted leading to lower kernel weight. Additionally, previous work has shown that glyphosate application, which leads to the inhibition of the shikimic acid pathway, could affect the carbon flow, which could also impact grain filling, during which starch is synthesized (Zobiole et al. 2011). As for the cultivar x treatment interaction, the lowest 1000-kernel weight was seen in the soft dough treatment for both cultivars, indicating that early application could have negative effects on kernel weight as previously found for treatment effects. However, the location x
Table 2.1. Average values for kernel, milling and flour quality characteristics for different glyphosate treatments

<table>
<thead>
<tr>
<th>Effect</th>
<th>1000 KW (g)</th>
<th>Vitreousness (%)</th>
<th>Wheat protein (12% m.b)</th>
<th>Falling number (seconds)</th>
<th>Extraction (%)</th>
<th>Flour protein (14% m.b)</th>
<th>Wet gluten (%)</th>
<th>Gluten index (%)</th>
<th>Flour ash (14% m.b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatmentb</td>
<td></td>
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<td></td>
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<td></td>
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<tr>
<td>Con</td>
<td>32.1 a</td>
<td>79 a</td>
<td>13.9 a</td>
<td>403 b</td>
<td>68.5 a</td>
<td>13.3 a</td>
<td>34.9 a</td>
<td>82 b</td>
<td>0.54 a</td>
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<tr>
<td>RP</td>
<td>31.8 a</td>
<td>72 a</td>
<td>13.9 a</td>
<td>417 a</td>
<td>67.8 b</td>
<td>13.3 a</td>
<td>35.1 a</td>
<td>79 b</td>
<td>0.54 a</td>
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<tr>
<td>SD</td>
<td>30.1 b</td>
<td>74 a</td>
<td>13.6 b</td>
<td>422 a</td>
<td>67.9 b</td>
<td>13.1 a</td>
<td>33.4 b</td>
<td>86 b</td>
<td>0.54 a</td>
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<td>Cul x Trtc</td>
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<tr>
<td>Glenn x Con</td>
<td>30.4 c</td>
<td>89 a</td>
<td>14.7 ab</td>
<td>387 d</td>
<td>67.4 c</td>
<td>14.0 a</td>
<td>36.9 a</td>
<td>85 ab</td>
<td>0.54 b</td>
</tr>
<tr>
<td>Glenn x RP</td>
<td>30.2 c</td>
<td>76 abc</td>
<td>14.7 a</td>
<td>406 c</td>
<td>66.6 d</td>
<td>13.9 a</td>
<td>37.1 a</td>
<td>82 bc</td>
<td>0.53 b</td>
</tr>
<tr>
<td>Glenn x SD</td>
<td>28.9 d</td>
<td>82 ab</td>
<td>14.3 b</td>
<td>413 bc</td>
<td>67.1 cd</td>
<td>13.7 a</td>
<td>35.1 b</td>
<td>90 a</td>
<td>0.53 b</td>
</tr>
<tr>
<td>Pros x Con</td>
<td>33.8 a</td>
<td>68 bc</td>
<td>13.2 c</td>
<td>419 abc</td>
<td>69.7 a</td>
<td>12.6 b</td>
<td>32.9 c</td>
<td>78 c</td>
<td>0.55 a</td>
</tr>
<tr>
<td>Pros x RP</td>
<td>33.4 a</td>
<td>67 c</td>
<td>13.2 c</td>
<td>428 ab</td>
<td>69.0 b</td>
<td>12.7 b</td>
<td>33.1 c</td>
<td>76 c</td>
<td>0.55 a</td>
</tr>
<tr>
<td>Pros x SD</td>
<td>31.3 b</td>
<td>65 c</td>
<td>12.9 c</td>
<td>430 a</td>
<td>68.8 b</td>
<td>12.5 b</td>
<td>31.7 d</td>
<td>82 bc</td>
<td>0.56 a</td>
</tr>
<tr>
<td>Loc x Trtd</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Car x Con</td>
<td>30.8 a</td>
<td>67 a</td>
<td>12.5 a</td>
<td>408 a</td>
<td>69.9 a</td>
<td>12.0 a</td>
<td>30.4 a</td>
<td>86 a</td>
<td>0.55 a</td>
</tr>
<tr>
<td>Car x RP</td>
<td>30.8 a</td>
<td>66 a</td>
<td>12.5 a</td>
<td>427 a</td>
<td>67.7 b</td>
<td>11.9 ab</td>
<td>30.1 a</td>
<td>84 a</td>
<td>0.53 a</td>
</tr>
<tr>
<td>Car x SD</td>
<td>30.7 a</td>
<td>79 a</td>
<td>12.2 a</td>
<td>427 a</td>
<td>68.1 b</td>
<td>11.6 b</td>
<td>29.4 a</td>
<td>90 a</td>
<td>0.53 a</td>
</tr>
<tr>
<td>Min x Con</td>
<td>36.3 a</td>
<td>89 a</td>
<td>14.3 a</td>
<td>407 a</td>
<td>68.9 b</td>
<td>13.5 a</td>
<td>35.6 b</td>
<td>80 a</td>
<td>0.52 a</td>
</tr>
<tr>
<td>Min x RP</td>
<td>36.0 a</td>
<td>87 a</td>
<td>14.4 a</td>
<td>409 a</td>
<td>68.5 b</td>
<td>13.7 a</td>
<td>37.1 a</td>
<td>72 b</td>
<td>0.52 a</td>
</tr>
<tr>
<td>Min x SD</td>
<td>36.0 a</td>
<td>88 a</td>
<td>14.3 a</td>
<td>409 a</td>
<td>69.9 a</td>
<td>13.7 a</td>
<td>36.5 a</td>
<td>75 ab</td>
<td>0.53 a</td>
</tr>
<tr>
<td>Pro x Con</td>
<td>29.3 a</td>
<td>80 a</td>
<td>15.0 a</td>
<td>394 b</td>
<td>66.8 a</td>
<td>14.3 a</td>
<td>38.7 a</td>
<td>79 b</td>
<td>0.57 a</td>
</tr>
<tr>
<td>Pro x RP</td>
<td>28.5 a</td>
<td>61 b</td>
<td>14.9 a</td>
<td>415 a</td>
<td>67.1 a</td>
<td>14.3 a</td>
<td>38.1 a</td>
<td>81 b</td>
<td>0.57 a</td>
</tr>
<tr>
<td>Pro x SD</td>
<td>23.7 b</td>
<td>54 b</td>
<td>14.3 b</td>
<td>429 a</td>
<td>65.8 b</td>
<td>13.9 b</td>
<td>34.4 b</td>
<td>93 a</td>
<td>0.57 a</td>
</tr>
</tbody>
</table>

aValues followed by the same letter are not significantly different at $P \leq 0.05$,
bCon: control, Cul: cultivar, Trt: treatment, Loc: location
cTrt: Treatment, Pros: cultivar Prosper
dLoc: location, Car: Carrington, Min: Minot, Pro: Prosper, LSD values show significant differences within each location at $P \leq 0.05$ significance level
treatment interaction was not significantly different between the treatments at two of the three locations. The 1000-thousand kernel weight was lower in the samples treated at soft dough stage only in the Prosper location.

Manthey et al. (2004), who also studied the effect of pre-harvest application of herbicides like glyphosate, paraquat and 2,4-D observed a decrease in 1000-kernel weight when glyphosate was applied at soft dough stage. Zollinger et al. (1999) and Darwent et al. (1994a) also reported that application of glyphosate prior to hard dough stage or physiological maturity causes decreases in kernel weight and kernel size. Similar observations, where pre-harvest desiccants caused negative effects on grain quality were found in other crops such as dry bean (Wilson and Smith 2002), sorghum seed (Baur et al. 1977), soybean (Bennett and Shaw 2000), peas (Baig et al. 2003) and rice (Bond and Bollich 2007). However, contrary to the findings of the current study, He et al. (2015) studied the impact of pre-harvest desiccants like diquat, paraquat and ethephan application of rice quality and reported that pre-harvest application did not affect grain quality traits such as 1000-kernel weight contrary to the results of the current study. Similar observations were made by Boudreaux and Griffin (2011) who studied soybean. Esfahani et al. (2012), found that pre-harvest desiccants like paraquat can cause minor reductions in oil content of rapeseed, without causing significant effects on yield and quality. Bellé et al. (2014) reported that glyphosate application at soft dough or ripe stages does not affect seed weight, however they found that seed germination is negatively impacted by early glyphosate application.

Wheat kernel vitreousness, is a measure of the compactness of starch granules in the protein matrix, and vitreous kernels have been found to be harder with higher protein content compared to non-vitreous kernels (Symons et al. 2003). Vitreousness has been identified as a factor impacting the extent of coarse particle size reduction during wheat milling (Greffeuille et
al. 2007). In this experiment, kernel vitreousness was not significantly different between the different glyphosate treated samples (Table 2.1). Significant differences were found for the cultivar x treatment interaction for kernel vitreousness. The values obtained for cultivar Glenn was generally higher in comparison to cultivar Prosper, however, within each cultivar, the effect of treatment did not show a clear trend. For example, the value for control was highest in both cultivars, however, the lowest was observed in the samples treated at ripe stage in cultivar Glenn, and soft dough stage in cultivar Prosper. Thus, significant differences between cultivars observed for vitreousness could be why this interaction was significant. The location x treatment interaction also did not show similar trends in the three locations, indicating that glyphosate application does not affect kernel vitreousness in different locations. For example, vitreousness was highest in soft dough in Carrington and Minot, whereas it was lowest in Prosper. As previous studies have shown, vitreousness is mainly impacted by environmental conditions, such as the availability of water and nitrogen as well as temperature, during grain maturation (Marzec et al. 2011). Thus, pre-harvest glyphosate application may not have an effect on kernel vitreousness.

Wheat protein content and composition (glutenin to gliadin ratio) are important parameters affecting end-use quality (Uthayakumaran et al. 1999). Wheat protein content (determined as a percentage), which is a measure of the amount of protein in the whole kernel, was significantly different between the treatment, where samples treated at soft dough showed significantly lower values compared to ripe and control (Table 2.1). This could be because protein deposition is interrupted by glyphosate when applied relative early during the maturation phase, leading to decreases in protein production (Preston et al. 1991). As for the cultivar x treatment interaction, cultivar Prosper showed significantly lower values for wheat protein content, in comparison to cultivar Glenn. However, for both cultivars, the lowest wheat protein content, in comparison to cultivar Glenn. However, for both cultivars, the lowest wheat protein
content was observed in the samples treated at soft dough. With reference to each location, Carrington and Minot did not show significant differences between the treatments, nevertheless in the location Prosper, the wheat protein content was lower in soft dough treated samples when compared to ripe and the control. Previous work on wheat proteins have shown that genotype, environment and the interaction can affect protein content as observed in the present study (Triboï et al. 2003; Uhlen et al. 1998).

Falling number is used to determine the effect of alpha-amylase activity in wheat (Perten 1964). Increased alpha-amylase activity could be caused by pre-harvest sprouting triggered by events such as untimely rain fall. In this study, falling number was higher in the treated samples in comparison to the control. The cultivar x treatment interaction was also significant for falling number, and in both cultivars the lowest value was found in the control (Table 2.1). At locations Carrington and Minot, significant differences were not observed for falling number; however in Prosper, the control showed a significantly lower values than the treated samples. Although the results suggest that glyphosate may cause increased falling number, which is indicative low alpha-amylase activity, it is unclear how glyphosate could cause such an impact. However, previous work has shown that in addition to pre-harvest sprouting, late maturity alpha-amylase and retained pericarp alpha-amylase could cause low falling numbers (Mares and Mrva 2008). Genes corresponding to late maturity alpha-amylase, appear to be genetic defects and are found in particular genotypes. These genes are subjected to complex modulation by environmental factors which further complicates its activity. Additionally, genotype and environment also affect the alpha-amylase activity in wheat (Johansson 2002).

Grain texture affects wheat milling and end-use quality characteristics, which include, milling yield, flour particle size and starch damage (Hogg et al. 2005). Wheat grain texture is
determined by two genes, puroindoline a and puroindoline b, which are found at the Hardness (Ha) locus. Variations in the abundance and activity of these puroindolines have been shown to affect milling quality. Milling yield is important economically as higher yield is beneficial for the industry. Milling quality was determined by percent extraction in this study. This parameter was lower in the treated samples compared to the control (Table 2.1). For the cultivar x treatment interaction, percent extraction was somewhat lower in cultivar Glenn compared to cultivar Prosper; however, the cultivars showed different trends for the different glyphosate treatments. At the different locations, percent extraction did not show a similar trend for the different treatments. That is, percent extraction was not lowest in the control in all locations. It is somewhat difficult to elucidate why pre-harvest glyphosate application could lower percent extraction, however it is possible that changed in the carbon flow caused by shikimic acid accumulation could affect grain texture leading to lower extraction. Effects of glyphosate on starch-protein interactions could also be a contributing factor. However, further studies are needed to firmly establish this association.

Several flour quality characteristics were also impacted by pre-harvest glyphosate application as shown in Table 2.1. Flour protein content, which is the protein content of refined flour, did not show significant differences between treatments, although flour protein content in the soft dough treated samples was a little lower than at ripe and control, similar to the observations made for wheat protein content. As mentioned earlier, wheat protein content and composition are important determinants of end-use quality, and it is interesting that pre-harvest glyphosate application does not affect protein content. As for the cultivar x treatment interaction, flour protein content was significantly lower in cultivar Prosper compared to cultivar Glenn, which highlights the influence of genotype on flour protein content as found in previous studies.
Additionally, flour protein content was significantly different at the different locations highlighting the effect of environmental conditions. However, at two locations (Minot and Prosper), grain from wheat treated at the soft dough stage showed the lowest flour protein content, which indicates that glyphosate could cause significant differences in protein content depending on environmental conditions.

Gluten proteins are composed of gliadin and glutenin proteins, which together impact visco-elastic properties of wheat dough (Bonomi et al. 2013). Early studies on wheat quality identified gluten content estimation as an important test for determining wheat quality (Kulkarni et al. 1987). In this study, wet gluten, which is representative of gluten content, was significantly lower in the soft dough treated samples compared to the ripe treated samples and the control (Table 2.1). In the analysis of cultivar x treatment interactions, wet gluten was significantly lower in cultivar Prosper compared to Glenn, and in both cultivars the lowest was observed in the soft dough treated samples. Wet gluten was also significantly different between locations; however, the treatments did not show the same trend at all three locations. For example, in Carrington, significant differences were not observed between the different treatments, however the control showed the lowest value in Minot, whereas soft dough treated samples showed the lowest value in Prosper. The differences in wet gluten caused by pre-harvest application of glyphosate could be due to the impact of glyphosate on gluten protein chemistry. Although, protein content is not affected by glyphosate, the inhibition of the shikimic acid pathway and the accumulation of shikimic acid could impact the chemical properties of gliadin and glutenin proteins, which are deposited late in the grain filling process, compared to proteins such as albumins and globulins, which are deposited early (Stone and Savin 1999).
In contrast to the observations for wet gluten, gluten index, which is indicative of protein quality, was highest in the soft dough treated samples, and lowest in the ripe treated samples (Table 2.1). Manthey et al. (2004) also reported that gluten index was increased two to eight units when glyphosate was applied at soft dough and ripe stages. Thus, the findings for protein content are in agreement with those of Manthey et al. (2004). Gluten index was significantly lower in cultivar Prosper compared to Glenn, and in both cultivars the highest gluten index was found in the treatment at soft dough stage. As for the location x treatment interaction, gluten index was highest at soft dough at locations Carrington and Prosper. Previous work on factors affecting gluten index have reported that genotype is the most important factor determining gluten index, while environmental factors and agricultural practices have a substantial effect gluten index (Gil et al. 2011). Glyphosate may cause significant differences in gluten index by affecting the deposition of glutenin and gliadin proteins as mentioned earlier. The ratio of glutenin to gliadin is critical in deciding dough and baking quality, thus changes in this ratio may translate into effects on end-use quality characteristics.

Flour ash content measures the mineral content of wheat flour, and traditionally, it has been a measure of flour purity and low ash content is preferred (Hinton 1959). In this study, flour ash did not show significant differences between treatments (Table 2.1). The ash content was somewhat lower in cultivar Glenn compared to Prosper; however, the values were within a very narrow range (0.53-0.56). The location x treatment interaction was not significant for this characteristic.

In this study, all kernel, milling and flour quality characteristics were significantly ($P \leq 0.05$) affected by cultivar and location. Many studies have discussed the effect of genotype, environment and the genotype-environment interaction on kernel quality traits. For example,
Bhatta et al. (2017) and Mladenov et al. (2001) determined that end-use quality traits are influenced by genotype, environment and their interaction. Similar observations were made by Fowler and Roche (1975) who reported that a large environmental effect can be observed concerning wheat yield, protein and protein related parameters. Moreover, Horvat et al. (2015) who studied the distribution of protein components in different genotypes and environments determined that growing year has a significant effect of crude protein content, total gliadin and total high molecular weight and low molecular weight glutenin proteins, and that the gliadins to glutenins ratio was affected by genotype and year at the same extent. In this context, the results of the current study align with previous findings where genotype and location are determining factors for kernel and flour quality traits.

2.4.2. Effect of glyphosate on dough quality

Farinograph measurements are indicative of dough properties and are also useful in predicting how flour will behave in bread and other baked goods. In this study, farinograph analysis was used to determine the effect of pre-harvest glyphosate application on dough quality characteristics. These results are presented in Table 2.2.
Table 2.2. Dough quality parameters as affected by treatment and other interactions\textsuperscript{a}

<table>
<thead>
<tr>
<th>Quality trait</th>
<th>Absorption (14% m.b.)</th>
<th>Peak Time (min)</th>
<th>Stability (min)</th>
<th>MTI</th>
<th>Quality number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment\textsuperscript{b}</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Con</td>
<td>62.2 a</td>
<td>6.3 a</td>
<td>7.1 b</td>
<td>37 a</td>
<td>103 b</td>
</tr>
<tr>
<td>RP</td>
<td>62.2 a</td>
<td>6.0 a</td>
<td>7.0 b</td>
<td>41 a</td>
<td>100 b</td>
</tr>
<tr>
<td>SD</td>
<td>61.6 b</td>
<td>5.9 a</td>
<td>8.3 a</td>
<td>31 b</td>
<td>108 a</td>
</tr>
<tr>
<td>Cul x Trt\textsuperscript{c}</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glenn x Con</td>
<td>62.8 a</td>
<td>7.0 a</td>
<td>8.0 b</td>
<td>33 b</td>
<td>116 b</td>
</tr>
<tr>
<td>Glenn x RP</td>
<td>62.8 a</td>
<td>6.8 a</td>
<td>7.9 b</td>
<td>40 a</td>
<td>112 b</td>
</tr>
<tr>
<td>Glenn x SD</td>
<td>62.3 b</td>
<td>7.1 a</td>
<td>9.5 a</td>
<td>29 b</td>
<td>122 a</td>
</tr>
<tr>
<td>Pros x Con</td>
<td>61.6 c</td>
<td>5.5 b</td>
<td>6.1 d</td>
<td>42 a</td>
<td>89 c</td>
</tr>
<tr>
<td>Pros x RP</td>
<td>61.6 c</td>
<td>5.3 bc</td>
<td>6.1 d</td>
<td>42 a</td>
<td>87 c</td>
</tr>
<tr>
<td>Pros x SD</td>
<td>60.9 d</td>
<td>4.8 c</td>
<td>7.0 c</td>
<td>33 b</td>
<td>93 c</td>
</tr>
<tr>
<td>Loc x Trt\textsuperscript{d}</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Car x Con</td>
<td>60.3 a</td>
<td>5.8 a</td>
<td>7.6 ab</td>
<td>37 ab</td>
<td>100 a</td>
</tr>
<tr>
<td>Car x RP</td>
<td>60.4 a</td>
<td>5.4 ab</td>
<td>7.2 b</td>
<td>39 a</td>
<td>91 b</td>
</tr>
<tr>
<td>Car x SD</td>
<td>60.5 a</td>
<td>5.0 b</td>
<td>8.1 a</td>
<td>30 b</td>
<td>97 ab</td>
</tr>
<tr>
<td>Min x Con</td>
<td>64.5 ab</td>
<td>6.4 a</td>
<td>6.3 a</td>
<td>37 b</td>
<td>103 a</td>
</tr>
<tr>
<td>Min x RP</td>
<td>64.7 a</td>
<td>6.1 a</td>
<td>5.8 a</td>
<td>51 a</td>
<td>96 a</td>
</tr>
<tr>
<td>Min x SD</td>
<td>64.4 b</td>
<td>6.2 a</td>
<td>6.4 a</td>
<td>37 b</td>
<td>103 a</td>
</tr>
<tr>
<td>Pro x Con</td>
<td>61.8 a</td>
<td>6.6 a</td>
<td>7.2 b</td>
<td>38 a</td>
<td>105 b</td>
</tr>
<tr>
<td>Pro x RP</td>
<td>61.5 a</td>
<td>6.6 a</td>
<td>8.0 b</td>
<td>34 ab</td>
<td>111 b</td>
</tr>
<tr>
<td>Pro x SD</td>
<td>59.9 b</td>
<td>6.7 a</td>
<td>10.4 a</td>
<td>25 b</td>
<td>123 a</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Values followed by the same letter are not significantly different at $P \leq 0.05$, MTI: Mixing tolerance index
\textsuperscript{b}Con: control, Cul: cultivar, Trt: treatment, Loc: location
\textsuperscript{c}Trt: treatment, Pros: cultivar Prosper
\textsuperscript{d}Loc: location, Car: Carrington, Min: Minot, Pro: Prosper, LSD values shows significant differences within each location at $P \leq 0.05$ significance level

Farinograph absorption, which indicates the optimum amount of water needed for processing wheat flour, was significantly lower at soft dough compared to the control and ripe, and absorption was somewhat higher in cultivar Glenn compared to Prosper (Table 2.2). In each cultivar, the lowest absorption was found in the samples treated at soft dough. As for the location x treatment interaction, Carrington did not show significant differences for the treatment, whereas the lowest values were observed in the soft dough treated samples at locations Minot and Prosper. Previous studies on factors affecting farinograph absorption have shown that
increasing particle size, protein content, starch damage and pentosan content increase absorption (Wu 2014). Decreased wheat hardness, protein content and pentosan content have been shown to have the opposite effect. In the analysis of kernel quality characteristics, minor differences were observed in protein content. Although not significant, these differences may have caused the differences in absorption. However, as previously stated, shikimic acid accumulation as a result of glyphosate application affects the carbon flow, thus the production of pentosans may be affected. Therefore, it is possible that this could also lead to decreases in absorption.

In general, farinograph peak time, stability, mixing tolerance index (MTI) and quality number are indicators of dough behavior and dough strength. Farinograph peak time, which is an indicator for optimum mixing time, did not show significant differences for treatments (Table 2.2). In the cultivar x treatment interaction, cultivar Glenn generally showed higher values compared to Prosper, and in the location x treatment interaction, differences in treatments was only observed in Carrington, which showed the lowest value for the samples treated at soft dough. Farinograph stability indicates how stable the dough is to over-mixing and dough strength, and higher values are favorable. In this experiment, the highest stability was reported in the soft dough treated samples. Similar to peak time, for the cultivar x treatment interaction, Glenn showed higher stability compared to Prosper, and variations in stability was most prominent in the Carrington location. Mixing tolerance index (MTI), which indicates the degree of softening during mixing, was lowest in the soft dough treated samples. As for the cultivar x treatment interaction, cultivar Glenn showed lower values compared to Prosper, and as for the location x treatment interaction, the lowest MTI was observed in samples treated at soft dough in all three locations. Higher farinograph quality number is favorable as it indicates strong dough. In this study, the highest quality number was reported in the soft dough treated samples, which is
in agreement with the observations made for stability and MTI. Additionally, the observations for cultivar \( x \) treatment and location \( x \) treatment were in line with the findings for stability and MTI.

Manthey et al. (2004) reporting similar findings, where dough mixing stability was increased when glyphosate was applied at soft dough or ripe stages of physiological maturity. This could be due to changes in protein composition and chemistry that is caused by glyphosate through the inhibition of the shikimic acid pathway and the effects of glyphosate on the carbon flow. Gliadin and glutenin proteins, which together make gluten proteins have different effects on dough behavior. Uthayakumaran et al. (2001) and Khatkar et al. (2002) showed that gliadins generally have a negative effect on dough properties. However, Huebner and Bietz (1986) and Park et al. (2006) found evidence supporting that gliadins have a favorable effect. The role of individual gliadin types (\( \alpha/\beta^{-} \), \( \gamma \) and \( \omega \)) is not yet clear. As for glutenin proteins, Khatkar et al. (1995) and Uthayakumaran et al. (1999) reported that these proteins are favorable for dough strength and that the ratio between glutenins and gliadins is a critical factors determining dough quality. In this context, glyphosate may cause changes to the deposition of gliadin and glutenin proteins, which may alter the chemistry and/or the ratio of these proteins leading to favorable dough properties.

All farinograph parameters were significantly affected by cultivar and location, and interaction between cultivar \( x \) location was significant for farinograph absorption and stability (Table 2.2). In this study, dough quality parameters were affected by genotype, environment and their interaction. Baker and Kosmolak (1977) determined that genotype-environment interactions were influential in determining dough quality traits determined by mixograph analysis and falling number, less important in determining farinograph traits such as absorption, and least
important in determining flour protein. As mentioned earlier, according to Horvat et al. (2015) genotype and growing year and location (environment) have significant effects on protein composition. Therefore, the differences in gliadin and glutenin protein compositions caused by genotype and environmental conditions could be the reason why cultivar and location were significant for all farinograph dough quality parameters. Moreover, Mladenov et al. (2001) found that farinograph absorption, peak time and quality number are significantly affected by the genotype, environments and the interaction of these factors.

2.4.3. Effect of glyphosate on baking quality

In this study, baking quality was also evaluated to determine how breadmaking is affected by pre-harvest application of glyphosate. The results of this analysis are presented in Table 2.3. In the analysis of baking quality, glyphosate treatment affected bake absorption, mix time, loaf volume and crumb color. Bake absorption was significantly lower in the control and soft dough treated samples compared to the treatment at ripe stage. In the farinograph analysis, the lowest absorption was found in the soft dough treatment, therefore the reason for this is not clear. Additionally, mix time was highest when samples were treated at soft dough, which can be explained with the observations made for dough quality. In the farinograph analysis, the soft dough treated samples showed highest values for stability and quality number indicating good dough quality, which may have resulted in increasing the bake mix time. Loaf volume was significantly higher in the treated samples (both ripe and soft dough) compared to the untreated control. In the previous section, dough quality was significantly affected when glyphosate was applied at soft dough, therefore it is reasonable that loaf volume was higher at soft dough. However, dough quality was not significantly affected at ripe stage, therefore it is unclear why loaf volume was significantly higher when treated at ripe compared to the control. Crumb color
was significantly different between the control and ripe, which may be due to processing differences.

In contrast to the observations made for loaf volume in the present study, Manthey et al. (2004) and Darwent et al. (1994a) reported that pre-harvest herbicide treatments do not affect loaf volume. McNeal et al. (1973) also reported that pre-harvest desiccants do not affect milling and baking quality of hard red spring wheat. Significant differences that were observed in loaf volume can also be attributed to the significant differences observed in gluten index, (where gluten index was highest at soft dough stage) since loaf volume is directly related to the quality and composition of wheat gluten proteins (Malalgoda et al. 2018). On the other hand, other breadmaking quality characteristics may not have been significantly affected by glyphosate treatment because the gluten index for all samples were within the range of 75-90, which as Curic et al. (2001) described, is within the optimum range for obtaining good quality bread. In addition to treatments, baking quality traits were also impacted by cultivar and location, and as previously explained this could be due to changes in protein quality that is caused by genotypic and environmental effects. As Roisch and Lingens (1980) and Gélinas et al. (2018) reported glyphosate does not have a significant impact on yeast activity, and this maybe the reason as to why most baking quality traits were not significantly affected by glyphosate.

Interactions between cultivar x treatment also had significant effects on baking quality (Table 2.3). In general, some quality traits showed significantly higher or lower values in specific cultivars, highlighting the effect of genotype. For example, cultivar Glenn showed higher loaf volume compared to cultivar Prosper. For the different cultivars, bake absorption did not show a specific trend. Mix time, as previously mentioned, was highest when treated at soft dough in both cultivars. Dough optimization score did not show a clear trend. Loaf volume was
highest at soft dough for cultivar Glenn and highest at ripe in cultivar Prosper. Therefore, there could be a genotype specific response in the case of this parameter. Symmetry, crust color, grain and texture, crumb color, crumb texture, fermentation height, oven-rise and specific volume were for the most part not significantly different for the cultivar x treatment interaction. As for the location x treatment interaction, most of the baking quality parameters identified did not show significant differences. However, for loaf volume, the highest value was observed in the soft dough treated samples in all three locations. Crumb texture was also affected by location as Carrington generally showed higher scores compared to the other two locations.

Same as for kernel and dough quality traits, baking quality traits are also influenced by genotype and environment as previous studies have found. For example, Hristov et al. (2010) reported that protein content, sedimentation value and loaf volume are affected by location. Similarly, Mladenov et al. (2001) determined that loaf volume and bake score are impacted by genotype, environments, and the interaction in winter wheat cultivars. Peterson et al. (1998) studied the effect of environment of baking quality of hard red spring wheat and found that that variations accounted by environmental effects was greater than that caused by genotype for dough and baking quality traits.
Table 2.3. Baking quality parameters as affected by treatment and the interaction between treatment and other factors^a

<table>
<thead>
<tr>
<th>Effect</th>
<th>Absorption</th>
<th>Mix time</th>
<th>Dough optimization</th>
<th>Volume</th>
<th>Symmetry</th>
<th>Crust color</th>
<th>Grain and texture</th>
<th>Crumb color</th>
<th>Crumb texture</th>
<th>Fermentation height</th>
<th>Over rise</th>
<th>Specific volume</th>
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<tr>
<td>Treatment^b</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Con</td>
<td>69.9 b</td>
<td>3.3 b</td>
<td>8.4 a</td>
<td>997 b</td>
<td>8.4 a</td>
<td>9.3 a</td>
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<td>75.7 a</td>
<td>8.1 a</td>
<td>3.4 a</td>
<td>7.7 a</td>
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<tr>
<td>RP</td>
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<td>3.4 b</td>
<td>8.7 a</td>
<td>1029 a</td>
<td>8.4 a</td>
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<td>68.7 a</td>
<td>8.2 a</td>
<td>3.5 a</td>
<td>7.9 a</td>
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<td>8.8 a</td>
<td>1028 a</td>
<td>8.9 a</td>
<td>9.5 a</td>
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<td>3.4 bc</td>
<td>9.0 ab</td>
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<td>7.8 c</td>
<td>926 c</td>
<td>7.7 c</td>
<td>8.8 b</td>
<td>7.1 b</td>
<td>7.6 b</td>
<td>91.1 a</td>
<td>8.1 a</td>
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<td>3.4 bc</td>
<td>8.3 bc</td>
<td>992 b</td>
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<td>9.1 b</td>
<td>7.3 ab</td>
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<td>8.2 c</td>
<td>977 b</td>
<td>8.6 ab</td>
<td>9.1 b</td>
<td>7.3 ab</td>
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<td>3.2 bc</td>
<td>7.5 bc</td>
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<td>8.2 a</td>
<td>3.6 a</td>
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<td>8.2 a</td>
<td>4.0 a</td>
<td>8.4 a</td>
</tr>
</tbody>
</table>

^aValues followed by the same letter are not significantly different at $P \leq 0.05$.
^bCon: control, Cul: cultivar, Trt: treatment, Loc: location
^cTrt: treatment, Pros: cultivar Prosper
^dLoc: location, Car: Carrington, Min: Minot, Pro: Prosper, LSD values shows significant differences within each location at $P \leq 0.05$ significance level
Overall, this study indicates that certain wheat quality characteristics such as, 1000-kernel weight, wheat protein content, percent extraction, wet gluten, gluten index, farinograph parameters, bake mix time and loaf volume are impacted by pre-harvest glyphosate treatment. In general, the effects were more pronounced when glyphosate was applied at soft dough compared to application at ripe stage. In a spiking experiment that was conducted (data not shown), where analytical grade glyphosate was spiked at 0, 15, 30 and 45 ppm to flour assumed to be free of glyphosate (organic flour and control flour from the field study), farinograph characteristics, such as stability showed a negative association with increasing glyphosate levels. This observation trend may have been caused by interactions between glyphosate and components in flour or dough. As determined by Worthing and Walker (1987) and MacBean (2008), glyphosate is soluble in water at 12g/l and 10.5g/l respectively, which indicates that chemical interactions could occur between glyphosate and water molecules. However, baking quality characteristics such as, loaf volume, grain and texture and crumb color did not show significant differences between the different spiked glyphosate levels.

2.5. Conclusions

In this study, two hard red spring wheat cultivars were grown in three locations across North Dakota and sprayed with glyphosate at soft dough and ripe stages of physiological maturity to determine the effect of glyphosate on wheat quality traits. Quality analyses were performed according to AACCI approved methods, and the results were analyzed using statistical tools. In line with previous findings regarding the effect of genotype and location/environment on wheat quality, the results of the current study also showed that cultivar, location and their interaction have significant effects on wheat quality traits ranging from kernel quality to baking quality. Glyphosate treatment timing was significant for kernel quality traits.
such as 1000-kernel weight, falling number, wheat protein content, percent extraction, wet gluten and gluten index. Thousand kernel weight and protein related characteristics were profoundly affected when glyphosate was applied at soft dough stage indicating that early application could lead to decreases in weight, however, gluten index was improved by early application possibly due to effects of glyphosate on the disruption of protein deposition and interference in the carbon flow. There was a general improvement in dough quality parameters by early application of glyphosate as indicated by farinograph stability, MTI and quality number. These observations can also be linked to changes in protein composition by glyphosate as a result of the disruption of protein deposition pathways and effects of shikimic acid accumulation. Loaf volume was found to be higher in the treated samples (both soft dough and ripe) in comparison to the control. However, most of the other baking quality traits were not significantly affected by glyphosate treatment. Thus, the results of this study indicate that the biochemical and compositional properties of wheat starch and proteins may be impacted by glyphosate application, possibly by the disruption of biochemical pathways responsible for starch and protein deposition, which results in changes in wheat quality characteristics as observed in this study. Additionally, application at soft dough stage caused more prominent effects on quality characteristics compared to application at ripe stage according to the results of this study.

2.6. References


CHAPTER 3. PRE-HARVEST GLYPHOSATE APPLICATION DURING WHEAT CULTIVATION: EFFECTS ON WHEAT STARCH AND PROTEIN PROPERTIES

3.1. Abstract

The herbicide glyphosate is sometimes used as a pre-harvest desiccant during wheat cultivation. Although glyphosate is an effective herbicide, side effects on crop physicochemical characteristics have been reported. Thus, the objective of this study was to determine how the timing of glyphosate application affects the physiochemical properties of starch, protein, and shikimic acid content in spring wheat. For this purpose, two wheat cultivars were grown in three locations, and glyphosate was applied at soft dough stage and ripe stage. An untreated control was also included. Upon harvest, starch properties, such as amylose and amylopectin ratio and molecular weight, starch pasting properties, granule size distribution and digestibility were determined using HPLC, RVA, mastersizer laser particle size analyzer and Englyst assay, respectively. Protein composition was examined using SE-HPLC, and shikimic acid content was quantified using HPLC. Significant ($P \leq 0.05$) differences were not detected between the different treatments and the control for amylose and amylopectin molecular weight. The proportion of B-type starch granules was lower in the treated samples than in the control, and vice versa for A-type granules. The Englyst assay indicated that rapidly digestible starch content was highest in the ripe application treatment, and lowest in the control, and vice versa for slowly digestible starch. Total starch content was lowest in the control, and resistant starch content was higher in the control compared to the treated samples. Flour pasting properties such as, RVA peak viscosity and final viscosity were significantly ($P \leq 0.05$) higher in soft dough treated samples compared to when glyphosate was applied at the ripe stage and the control. However, significant differences were not observed for starch pasting properties. The proportion of
different gluten protein fractions was not affected by glyphosate treatment. As for shikimic acid accumulation, the greatest accumulation was at soft dough stage, and the accumulation decreased from ripe to control. In this context, glyphosate application time appears to have an influence on starch characteristics of spring wheat, especially on starch granule distribution, flour pasting properties, and digestibility as well as shikimic acid accumulation.

3.2. Introduction

Glyphosate is the most widely used herbicide in the world (Benbrook 2016). From 1996 to 2014, the use of glyphosate increased from 36 million kilograms to 747 kilograms. Introduction of glyphosate resistant crops and new agricultural practices such as the use of glyphosate as a harvest-aid are the main reasons contributing to the rapid increase in glyphosate use in agriculture in the United States and worldwide. The herbicidal mode of action of glyphosate arises from its ability to inhibit the enolpyruvylshikimate phosphate synthase (EPSPS) enzyme in the shikimic acid pathway, and as a result, the biosynthesis of aromatic amino acids such as phenylalanine, tyrosine and tryptophan is disrupted (Williams et al. 2000). The shikimic acid pathway is found in plants, fungi and bacteria, hence the exclusivity of the mode of action. Cajacob et al. (2004) also reported that the EPSPS enzyme’s active site is highly conserved in higher plants. In the case of wheat, glyphosate is sometimes used as a pre-harvest desiccant, and is recommended to be applied at ripe stage of physiological maturity. However, it is sometimes applied before crops reach this state to accelerate the maturation process and ensure timely harvest. However, as Savin and Slafer (1991) and Yan and Hunt (2001) showed, the stage of physiological maturation is important when crops come into contact with different environmental factors as well as agrochemicals.
As demonstrated in previous studies, glyphosate and its primary breakdown product aminomethylphosphonic acid (AMPA) can affect biochemical pathways in plants (Gomes et al. 2014). In a study where the impact of glyphosate on starch synthesis in relation to photosynthesis was investigated, it was found that glyphosate can reduce photosynthesis by way of inhibiting the biosynthesis of chlorophylls, fatty acids and amino acids (Fedtke and Duke 2005). The reduction in the synthesis of secondary metabolites such as quinones could lead to decreases in photosynthetic activity. Similar observations were made by Zobiole et al. (2012) who studied glyphosate-resistant soybeans. Zobiole et al. (2011) determined that glyphosate’s role in decreasing the amount of chlorophyll in leaves could lead to decreased rate of photosynthesis. Furthermore, glyphosate’s role as a metal chelator could be a contributing factor affecting photosynthesis rate in crops as it could have an impact on enzyme co-factors required for different biosynthetic pathways (Gomes et al. 2014). Cakmak et al. (2009) found that glyphosate binds to magnesium in leaves leading to decreased photosynthesis. Glyphosate could also affect key enzymes involved in carbon fixing such as, ribulose-1, 5-biphosphate (RuBP) and 3-phosphoglyceric acid, and the enzyme ribulose-1,5-biphosphate carboxylase, and also reduce starch biosynthesis as shown by (Servaites et al. 1987).

Wheat protein chemistry can also be affected by glyphosate due to the inhibition of the shikimic acid pathway. Craven et al. (2007) investigated how glyphosate application at soft dough and hard dough impacts wheat quality and determined that the impact varied among growing seasons and cultivars. In general, they observed that glyphosate application at soft dough stage decreased protein content. Mondal et al. (2009) made similar observations where glyphosate application had an inhibitory effect on total protein content. They also observed significant reductions in chlorophyll content, and the availability of Na+ and K+ ions. In peas,
Zulet et al. (2013) found that glyphosate inhibits the biosynthesis of amino acids by inducing specific proteolytic pathways. Foley et al. (1983) also found that glyphosate caused increased ATP levels in root tissue by inhibiting the biosynthesis of proteins. However, Darwent et al. (1994) reported that glyphosate application rate at grain moisture levels lower than 40% did not affect protein content in wheat.

Some studies have suggested that the shikimic acid accumulation as a result of glyphosate application could be a contributing factor causing plant death. Henry et al. (2007) studied shikimic acid accumulation in sunflower, wheat and proso millet and found that glyphosate application at lower levels leads to some accumulation although it may not be significantly different than the control, whereas application at higher rates caused greater accumulation. Kim et al. (2008) studied shikimic acid accumulation in grasses and broad leaf plants and determined that accumulation is stimulated at lower doses of glyphosate in grasses compared to broad leaf plants. In another study, Cardinali et al. (2015) measured shikimic acid accumulation to differentiate between glyphosate resistant and susceptible horseweed biotypes. With reference to wheat, Bresnahan et al. (2003) found that shikimic acid accumulated as a result of glyphosate application, and that shikimic acid accumulation peaked 3-7 days after treatment and then declined until harvest. They also determined that shikimic acid was transferred to subsequent baked products.

In this context, the objective of this study was to determine the effect of glyphosate on wheat starch properties, protein composition and accumulation of shikimic acid. For this purpose, glyphosate was applied at ripe and soft dough stages of physiological maturity, and harvested upon maturity, after which chemical analyses were performed.
3.3. Materials and methods

3.3.1. Field study design

A 3 x 3 x 2 factorial design (location x treatment x cultivar) with a split-plot layout was used for this study, where location was the main plot and treatment x cultivar was the sub-plot. Therein, two wheat cultivars, Glenn and Prosper, were grown at three locations, Minot, Carrington and Prosper in the state of North Dakota. Glyphosate was sprayed at the recommended rate 1.1 kg of active ingredient/ha at soft dough stage and ripe stage and harvested at physiological maturity. A control where glyphosate was not sprayed was also used in this study. Each treatment was repeated three times, bringing the total number of samples to 54. The samples were cleaned and milled in-house at the North Dakota State University by the Hard Red Spring Wheat Research group. White flour samples were used in the analyses described below.

3.3.2. Analysis of amylose to amylopectin ratio and molecular weight

For isolation of starch, approximately 30 to 40 mg of white flour was first defatted by adding 2.5 mL of methanol and heating at 100°C for 30 min. Afterwards, the samples were centrifuged at 2000 rpm for 5 min (Allegra X-12, Beckman Coulter, Indianapolis, IN, U.S.A), and the supernatant was decanted, and subsequently the pellet was dried at 55°C in an oven. Starch was extracted from the dried pellets by adding 2 mL of potassium hydroxide/urea solution (4.5 mL 1M KOH with 0.5 mL 6M urea) and heating at 100°C for 15 min. Then the starch was precipitated by adding 6 mL of 95% ethanol. The samples were then centrifuged at 2000 rpm for 5 min and dried overnight in an oven.

The defatted precipitated starch was prepared for SE-HPLC analysis by treating with KOH and urea as previously described by (Grant et al. 2002). The extracted starch was solubilized by adding 4.5 mL of 1M KOH and 0.5 mL of urea and heating at 100°C for 90 min.
After cooling the samples to room temperature, a 1 mL aliquot of each sample was neutralized (until measured pH was 7) with 1M HCl and filtered through a 0.45 µm nylon membrane (VWR International). An Agilent 1200 series HPLC system (Agilent Technologies, Santa Clara, CA, U.S.A.) which was connected to an auto sampler, refractive index (RI) detector, and a Wyatt Dawn Helios-II multi-angle light scattering (MALS) detector was used in this experiment. A Waters Ultrahydrogel guard column (6 µm, 6 mm x 40 mm), and Ultrahydrogel 1000 (12 µm, 7.8 mm x 300 mm) and a linear size exclusion column (10 µm, 7.8 mm x 300 mm) (Waters, Milford, MA, U.S.A.) were used for the separation of amylose and amylopectin. The experiment was run at 40°C at 0.4 mL/min and the injection volume was 20 µl. A mobile phase of HPLC grade water was used.

The ChemStation software (HP ChemStation for LC Rev. A.04.01) was used to determine the amylose to amylopectin ratio by integrating the respective peaks. The Astra 6.0.5 software (Wyatt Technology Corporation, Santa Barbara, CA, U.S.A.) was used to determine the molecular weight of amylose and amylopectin. During this analysis a dn/dc value of 0.146 was used as per the findings of You et al. (1999). The data was normalized using pullulan standards, after which baseline corrections and peak alignments were performed. The Debye model with a fit degree of 2 and a first-order polynomial fit were applied using the calculations.

3.3.3. Analysis of starch digestibility using Englyst assay

White flour samples were used to prepare bread according to the American Association of Cereal Chemists International (AACCI) method 10-09.01 with modifications (fermentation period was shortened to 2 hours, fungal amylase was used in place of malt powder, instant dry yeast was used instead of compressed yeast and 1 mL of 10% ammonium phosphate was added to each bread dough sample during mixing), and the bread samples were used to determine the in
vitro digestibility of starch according to a method previously described by Englyst et al. (1992). The 0.1M sodium acetate buffer, pH 5.2 and the 5 mg/mL glucose standards were prepared ahead of time. However, the enzyme solutions were prepared on the day of analysis.

Amyloglucosidase (140 AGU/mL), Megazyme International, Bray, Co. Wicklow, Ireland) was brought to 25 mL by adding deionized water. To prepare the invertase enzyme, 60 mg of powered invertase (Sigma I-4504) was added to 8 mL of deionized water. To prepare the pancreatin enzyme, 18 g of pancreatin (Sigma P-7545) was equally distributed between six centrifuge tubes and dispersed with 20 mL of deionized water in each tube. The tubes were shaken at 140 rpm at 4°C for 10 min in a MaxQ 400 shaker (Thermo Scientific, Marietta, OH, U.S.A.) and centrifuged at 3000 g for 10 min (Allegra X-12, Beckman Coulter, Indianapolis, IN, U.S.A.). Afterwards, approximately 108 mL of pancreatin supernatant was mixed with 12 mL of amyloglucosidase and 8 mL of invertase to create the enzyme solution used for the assay.

A volume of 20 mL of sodium acetate buffer was added to 300 mg of sample, 300 mg of white bread standard, 50 mg of glucose and 10 mL of water (blank). Five glass marbles and 50 mg of guar gum were added to each tube and agitated for 10 min so that the contents are dispersed. Subsequently 5 mL of the previously prepared enzyme solution was added to each tube at one minute intervals. Every 20 min, for a period of 180 min in total, 0.5 mL of aliquots were placed in tubes containing 5 mL of absolute ethanol. These tubes were stored overnight (about 16 hours) at 4°C.

The following day, the samples were centrifuged (Beckman Coulter, Indianapolis, IN, U.S.A.) at 1500 x g for 10 min. A volume of 0.1 mL of was taken from the sample supernatants, glucose standard solutions (0.1, 0.2, 0.5, 1.0, 2.0 and 5.0 mg/mL), and the blank (water). The glucose content in the samples were determined using the GOPOD assay kit from Megazyme
International, where 3.0 mL of GOPOD reagent was added to each tube and incubated in a water bath at 50°C for 20 min, after which the absorbance was determined at 492 nm using a microplate reader (Thermo Electron, Vantaa, Finland). A standard curve was prepared to determine the glucose content released. The rapidly digestible starch (RDS), slowly digestible starch (SDS) and resistant starch (RS) were determined from the glucose values at 20 and 120 min. That is, the RDS is considered as the hydrolyzed starch portion from 0-20 min, SDS is the hydrolyzed starch between 20 – 120 min and the RS is the remaining starch after 120 min of digestion (Englyst et al. 1992). The hydrolysis index (HI) was calculated by dividing the area under the hydrolysis curve of the sample by the area obtained for the white bread standard (0-180 min). The estimated glycemic index (eGI) was determined using the following equation, which was described by Granfeldt et al. (1992).

\[
eGI = 8.198 + (0.862 \times HI)
\]

### 3.3.4. Granule size analysis using mastersizer

In order to determine the granule size distribution of the samples, starch was first isolated from white flour samples by removing the gluten proteins. For each sample, 40 g of flour was used to extract starch. First, 20 g of flour was washed using a glutomatic (Glutomatic® Gluten Index, GM2200, Perten Instruments, Sweden). The flour slurry was then centrifuged at 1500 x g (Allegra X-12, Beckman Coulter, Indianapolis, IN, U.S.A) for 10 min, and the supernatant was discarded. The starch pellet was then washed with 200 mL of deionized water by resuspension and centrifuged as before. Next, the yellowish contaminated material was scraped off from the pellet. The water washing step was repeated in this manner. Following the water washing steps, the pellet was washed with 100 mL of 95% ethanol by resuspension, and left for 15 min, and centrifugation was performed as described earlier. Finally, the pellet was washed with 100 mL of
acetone and centrifuged again. The same procedure was repeated for the second 20 g of the sample. The starch pellets were then combined and dried overnight in an oven at 35°C. The following day, the dried starch pellet was ground using a mortar and pestle and sieved on a 75 µm sieve for 4 min. A Mastersizer 3000 with an Aero S apparatus (Malvern Panalytical, Malver, U.K.) was used to determine the starch granules size distribution. The gap distance was set at 2.5, and the analysis time was approximately 5 min. The obtained data was used to determine the percentage of A and B type starch granules.

3.3.5. Analysis of starch pasting properties using rapid visco analyzer (RVA)

The AACCI method 76-21.01 method was used to determine the pasting properties of white flour samples using a rapid visco analyzer (Perten RVA 4500, Perten Instruments, Hägersten, Sweden). The starch extracted as described in the previous section was also analyzed using RVA according to the same AACCI method. For wheat flour samples, 3.5 g of flour (adjusted to 14% m.b.) was used and for starch samples 3.0 g (adjusted to 14% m.b.) was used in the RVA analysis.

3.3.6. Protein composition and molecular weight analysis using SE-HPLC

A method previous described by Gupta et al. (1993) with modifications was used for protein extraction for SE-HPLC analysis Ohm et al. (2009). An extraction buffer composed of 0.5% SDS and 0.05 M sodium phosphate, pH 6.9 was used for the extraction of SDS-extractable and unextractable proteins. To obtain the extractable fraction, 1 mL of extraction buffer was added to 10 mg of flour (adjusted to 14% moisture content). The sample was stirred for 5 min using a vortex mixture at 2,500 rpm (Fischer Scientific, Waltham, MA, U.S.A.). The sample was then centrifuged at 20,000 x g (Eppendorf Centrifuge 5424) for 15 min, and the supernatant was filtered through a 0.45 µm PVDF membrane (Sun Sri, Rockwood, TN, U.S.A.). Immediately
afterwards, the protein extract was heated at 80°C for 2 min in a water bath to inactive any enzymes. The residue from the above extraction was used to obtain the SDS-unextractable protein fraction. A volume of 1 mL of extraction buffer was added to the residue and sonicated using a probe type sonicator at 10W power setting for 30 seconds (Sonic Dismembrator 100, Fisher Scientific, Waltham, MA, U.S.A.). Afterwards, centrifugation and filtration were performed as explained earlier. The SDS-extractable and unextractable proteins were analyzed separately using an Agilent 1100 series narrow bore size exclusion column (300 x 4.5 mm, BIOSEP SEC S4000, Phenomenex, Torrance, CA, U.S.A.) and guard cartridge (BIOSEP SEC S4000, Phenomenex) as described in Ohm et al. (2009). A solvent comprised of 50% acetonitrile in water with 0.1% (v/v) trifluoroacetic acid (TFA), with a flow rate of 0.5 mL/min was used for this analysis. The injection volume was 10 µL. An Agilent 1200 photodiode array detector (Agilent Technologies, Waldbonn, Germany) was used to read the signal at 214 nm.

The MATLAB software (2015, The MathWorks, Natick, MA, U.S.A.) and an in-house program was used to process the data as described in Ohm et al. (2009) and Malalgoda et al. (2018). The Astra software and the ChemStation analysis tools were used to determine changes in the proportion of different gluten protein fractions.

3.3.7. **Shikimic acid quantitation in whole wheat flour**

Shikimic acid was extracted from whole wheat flour samples according to the method of Zelaya et al. (2011). A volume of 2.0 mL of 0.25 N HCl was added to 0.2 g of flour and pulverized for five min using a homogenizer system (SCIENCEWARE® Wilmad LabGlass Micro Tube Homogenizer System, Bel-Art, VWR International, Radnor, PA. U.S.A.). Afterwards, the samples were sonicated for 1 hour in an ultrasonic sonicator (Branson 1200m Bransonic Ultrasonic Cleaner, Branson Ultrasonics Coorporation, Danbury, CT, U.S.A.), then
centrifuged at 10,000 x g for 15 min (Allegra 64R Centrifuge, Backman Coulter). The supernatants were then filtered through a 0.45 µm nylon membrane (VWR International, Radnor, PA, U.S.A).

The HPLC method for the quantitation was performed as previously described by Feng et al. (2004). A shikimic acid standard curve created using analytical grade shikimic acid (Sigma) in 0.25N HCl at concentrations of 10, 25, 50, 100 and 200 ppm was used for the quantification of shikimic acid. The blank or zero used in the analysis was 0.25 N HCl. A Phenomenex Lune 5µm C18 column (250 x 4.6 mm) (Phenomenex, Torrance, CA, U.S.A) was used in the analysis. Solvent A comprised on 6 mM phosphoric acid in water, and solvent B was methanol. Both solvents were filtered through a 0.2 µm filter prior to running on the instrument. The flow rate was 1 mL/min and the injection volume was 20 µL. The following solvent gradient was used during the experiment, 0 – 6 min: 0% B, 6 – 10 min: % B was increased to 100%, 10 – 20 min: % B was maintained at 100%, 20-21 min: % B was reduced to 0% and 21-30 min: 0% B. An Agilent 1100 variable wave length detector (Agilent Technologies) was used to detect the signal at 215 nm. To quantify the shikimic acid in the samples the ChemStation software was used.

3.3.8. Statistical analysis

The SAS software (Version 9.3, SAS Institute, Cary, NC) was used to perform Analysis of Variance (ANOVA) and mean separation using (Fisher’s protected LSD).

3.4. Results and discussion

3.4.1. Starch amylose to amylopectin ratio and molecular weight

Starch is the major storage compound in plants (Copeland et al. 2009). It is made of two polymers, amylose, a sparsely branched α(1→ 4) linked glucan and amylopectin, which is a highly branched structure composed of chains of α(1→ 4) glucose with α(1→ 6) branching
points. Starch molecular weight determination has been found to be a difficult task due to reasons such as difficulties in solubilization, separation based on size without degradation and broad size range (Gidley et al. 2010). One of the most important events in the process of baking is the gelatinization of starch (Goesaert et al. 2008). It has been suggested that the uptake of water during starch granule swelling results in the loss of flexibility of gluten proteins causing the destabilization of gas cells and their eventual rupture. Furthermore, crumb firmness appears to be determined by the rigidity of gelatinized granules and leached amylose. As for firmness during storage, this phenomenon seems to be determined by amylopectin retrogradation and strengthening of starch-starch and gluten-starch interactions. In this study, treatment did not cause significant differences in the percentage of amylose and amylopectin, as well as molecular weight (Table 3.1). Cultivar, location and the interaction of the different factors did not cause significant differences in the molecular weight of starch polymers. However, cultivar appeared to be a source of variation for the percentage of amylose and amylopectin. The interaction between factors also did not cause significant differences in the percentage of starch polymers.

Table 3.1. Starch amylose and amylopectin characteristics averaged across treatment, cultivar and locationa

<table>
<thead>
<tr>
<th>Effect</th>
<th>Percentage (%)</th>
<th>Molecular weight (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amylopectin</td>
<td>Amylose</td>
</tr>
<tr>
<td>Treatmentb</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>75.0 a</td>
<td>25.0 a</td>
</tr>
<tr>
<td>RP</td>
<td>75.4 a</td>
<td>24.6 a</td>
</tr>
<tr>
<td>SD</td>
<td>75.3 a</td>
<td>24.7 a</td>
</tr>
<tr>
<td>Cultivar</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glenn</td>
<td>75.6 a</td>
<td>24.4 b</td>
</tr>
<tr>
<td>Prosper</td>
<td>74.8 b</td>
<td>25.2 a</td>
</tr>
<tr>
<td>Location</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carrington</td>
<td>75.1 a</td>
<td>24.9 a</td>
</tr>
<tr>
<td>Minot</td>
<td>75.6 a</td>
<td>24.4 a</td>
</tr>
<tr>
<td>Prosper</td>
<td>75.1 a</td>
<td>24.9 a</td>
</tr>
</tbody>
</table>

a LSD for each location is given at \(P \leq 0.05\) significance level, where values followed by the same letter are not significantly different at \(P \leq 0.05\)

b RP: Ripe treatment, SD: soft dough treatment
In general, the amylose and amylopectin percentages were approximately 25% and 75% respectively (Table 3.1) in line with previous findings (Ball et al. 1998; Hurkman et al. 2003; Medcalf and Gilles 1965). As previously reported, differences in the percentage of amylose and amylopectin was found to be significantly difference between cultivars (Medcalf and Gilles 1965). Although there were variations in the molecular weight of starch polymers, as mentioned earlier, significant differences were not found. In early publications, the approximate weight of amylose and amylopectin was reported as 87,000 – 140,000 g/mole (Da) and 4 million g/ mole (Da) respectively (Bechtel et al. 1964). However, in recent years, with advancements in analytical technology, revised values have been published. For example, the molecular weight of amylopectin and amylose has been reported as 10-310 million Da and 0.1 – 0.2 million Da respectively (Ball et al. 1998; Simsek et al. 2013; Simsek et al. 2014; Yoo and Jane 2002). In the current study, the molecular weight for amylopectin ranged between 3 and 19 million Da, and for amylose it was between 1 and 3 million Da. The average molecular weight for amylopectin and amylose was 9.91 x 10^6 and 2.1 x 10^6 Da, respectively. Similar values for average molecular weight was reported by Schwebach (2016), however, the average molecular weight determined in the current study are somewhat low for amylopectin and high for amylose. However, given the broad range of molecular weights reported by Gidley et al. (2010), the values in the present study can be considered to be within range.

As for the effect of herbicides on wheat starch accumulation, Kumar (2012) determined that herbicides such as 2,4-D and isoproturon can cause decreases in carbohydrate accumulation in proportion to the level of application. Moreover, studies investigating the effect of glyphosate on photosynthesis, found that glyphosate causes decreases in chlorophyll synthesis leading to decreased accumulation of starch (Fedtke and Duke 2005; Zobiole et al. 2011; Zobiole et al.
Similar observations were made by Yin et al. (2008) with reference to the application of the herbicide isoproturon on wheat. Other studies have highlighted that glyphosate’s metal chelating ability could interfere with key enzymes in used in photosynthesis leading to decreases in starch accumulation (Cakmak et al. 2009; Gomes et al. 2014). However, there is no literature available discussing the role of glyphosate or other herbicides on the molecular weight of wheat starch polymers. In the present study, the non-significant results for starch molecular weight may have been found because glyphosate may not reduce the molecular weight of starch although total accumulation of starch in the wheat endosperm maybe decreased.

Several studies have found that environmental conditions can affect the molecular weight of wheat starch. For example, Simsek et al. (2014) studied the effect of pre-harvest sprouting on wheat starch molecular weight and determined that starch molecular weight was deceased as a result of increased α-amylase activity that occurred due to early sprouting. Furthermore, Hurkman et al. (2003) determined that high temperature during the grain filling phase of physiological development decreased the duration of starch accumulation in the wheat endosperm.

3.4.2. Starch digestibility as determined by the Englyst assay

Starch is a major component of wheat grains and constitutes to approximately 60-75% of the grain (Delcour and Hoseney 2010). Starch also provides majority (70-80%) of calories in the human diet. According to the classification of Englyst et al. (1992), the primary component of rapidly digestible starch (RDS) is amylopectin, whereas slowly digestible starch (SDS) is mostly composed of amylose. The remainder of starch is composed of indigestible or resistant starch (RS) components, which are subjected to fermentation by gut microbial organisms in the large intestine (Stone and Morell 2009). Englyst et al. (1992) described hydrolysis (HI) and estimated
glycemic index (eGI) as values indicating the amount of starch broken down by gut enzymes and the blood glucose response after consuming a specific food respectively. In this study, the estimated GI is reported as an *in vitro* procedure to mimic digestion was used.

RDS, SDS, RS and total starch (TS) showed significant ($P \leq 0.05$) differences for treatment (Table 3.2). RDS was highest in the ripe treated samples, and lowest in the control, and vice versa for SDS. Total starch was lowest in the control, and resistant starch was higher in the control compared to the treated samples. Cultivar caused significant differences in RDS, SDS and TS, while location caused significant differences in all Englyst assay parameters. The interaction between cultivar and location was significant for RDS, SDS, TS and RS. The cultivar x treatment interaction was significant for RDS and RS, whereas the location x treatment interaction was significant for RDS, SDS, TS, HI and eGI. The three-way interaction between cultivar, location and treatment was significant for all starch digestibility parameters determined using the Englyst assay.
The results shown in Table 3.2 indicate that the digestibility of starch was impacted by several factors including the application of glyphosate at different levels of physiological maturity. In the previous section, the results of this study indicated that glyphosate does not impact the molecular weight or the percentage of amylopectin and amylose. Therefore, although these parameters are not affected, glyphosate may cause structural or chemical changes to wheat starch leading to differences in TS, RDS, SDS and RS. As previously reported by Patil (2008), starch digestibility is affected by amylose-to-amylopectin ratio, particle size, starch-protein interactions, physical form, and method and time of cooking. As reported by Wang et al. (2015)
complex structural organizations in wheat starch that occurs during starch biosynthesis could impact starch digestibility. For example, arrangement of double helices, crystalline and amorphous regions, super helices, blocklets and growth rings into the semi-crystalline granules are factors affecting the susceptibility of starch to enzymatic digestion processes. Moreover, Dhital et al. (2017) described two main factors affecting starch digestibility as (i) barriers that slow or prevent enzymes from accessing starch and (ii) structural features of starch that slow or prevent amylase enzyme activity. From a different perspective, starch gelatinization which occurs during cooking, is affected by factors like the botanical origin of starch, type of starch, water availability, temperature, rate and duration of heating and the magnitude of shear forces. In additiona, the process of gelatinization is important because it affects food processing and human nutrition (glycemic index) (Wang and Copeland 2013). In this context, glyphosate may cause structural changes to starch molecules as a result of the disruption of the shikimic acid pathway and thereby interfere with the carbon flow of affected plants leading to changes in digestibility. Although there have not been many studies focused on understanding the effect of glyphosate timing on wheat starch digestibility, there have been indications that agrochemicals can affect starch characteristics. For example, Nitika et al. (2008) reported that wheat cultivars grown under organic conditions have significantly higher protein and starch digestibility compared to wheat grown under non-organic conditions.

3.4.3. Starch granule size

Starch granules range in size from 1 to 100 µm and can have different shapes (polygonal, spherical or lenticular). Bechtel et al. (1990) studied the development of starch granules during endosperm maturation and determined that that at maturity, A-type starch granules (diameter ≥ 15.9 µm) account for 4.8%, whereas B-type starch granules (5.3 – 15.9 µm) account for 49.5%.
Granules with diameter less than 5.3 µm was termed C-type and accounted for 45.7%. However, in later studies, a higher proportion of A-type granules and lower proportion of B-type granules were reported. For example, Zhang et al. (2016) found that the volume percentage of A-type starch granules range from 68.4 – 90.1%, and the percentage of B-type granules range from 7.2 – 25% in Chinese spring wheat cultivars. In this study, treatment, cultivar and location caused significant differences in the proportion and A and B type starch granules. As for interactions, the cultivar x location and location x treatment interactions were significant for the proportion of A-type granules, whereas the location x treatment was significant for the proportion of B-type granules. The three-way interaction between cultivar x location x treatment was not significant for both types of granules. Figure 3.1 shows the distribution of A and B type starch granules in the different treatments.

![Figure 3.1. Distribution of A and B type granules in the different glyphosate treatments](image)

According to Table 3.3, the values reported in this study fall within range for previously determined values for the proportion of different starch granules cited above. Glyphosate treatment significantly increased the proportion of A-type granules, while decreasing the proportion of B-type granules. Cultivar and location also caused significant differences in the proportion of granules.
Table 3.3. Effect of glyphosate on starch granule size

<table>
<thead>
<tr>
<th>Effect</th>
<th>Granule A (%)</th>
<th>Granule B (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment(^b)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>78.1 b</td>
<td>14.4 a</td>
</tr>
<tr>
<td>RP</td>
<td>79.4 a</td>
<td>13.7 b</td>
</tr>
<tr>
<td>SD</td>
<td>79.1 a</td>
<td>13.9 b</td>
</tr>
<tr>
<td>Cultivar</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glenn</td>
<td>79.6 a</td>
<td>12.6 b</td>
</tr>
<tr>
<td>Prosper</td>
<td>78.1 b</td>
<td>15.4 a</td>
</tr>
<tr>
<td>Location</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carrington</td>
<td>78.0 b</td>
<td>15.0 a</td>
</tr>
<tr>
<td>Minot</td>
<td>77.0 b</td>
<td>14.4 a</td>
</tr>
<tr>
<td>Prosper</td>
<td>81.5 a</td>
<td>12.6 b</td>
</tr>
</tbody>
</table>

\(^a\) LSD for each location is given at \(P \leq 0.05\) significance level, where values followed by the same letter are not significantly different at \(P \leq 0.05\)

\(^b\) RP: Ripe treatment, SD: soft dough treatment

The proportion of A and B type starch granules might be changed by glyphosate as a result of interfering in the deposition of starch. A-type starch granules are deposited early in grain filling, whereas B-type granules are synthesized later (Bechtel et al. 1990). Glyphosate may interfere with the deposition of B-type granules, resulting in higher proportion of A-type granules and lower proportion of B type granules compared to the control. The difference between A and B type starch granules lie in the composition, chain length distribution of amylopectin, relative crystallinity and microstructure, which refers to features such as surface pores, channels and cavities as summarized by Zhang et al. (2016). These differences result in variations in starch swelling properties, gelatinization, retrogradation and pasting properties. For example, the gelatinization enthalpy, amount of amylose and starch pasting properties are higher in A-type granules, whereas gelatinization onset and peak temperatures are lower. As for B-type granules, these granules have higher proportion of lipid-amylose complexes and swelling power, broader gelatinization temperatures and lower gelatinization enthalpy. Thus, glyphosate’s effect
in changing the distribution of starch granules can cause changes in the gelatinization properties of wheat starch. The current study serves as one of the first studies reporting the effect of glyphosate on wheat starch granule distribution since many of the previous work on glyphosate and wheat have focused on wheat quality parameters such as yield and seedling viability.

Other studies in the area of wheat starch have determined that genotype effects can cause significant differences in the proportion of the different starch granules, similar to the results of this study (Raeker et al. 1998). In this study, location also caused significant differences in the distribution of starch granules (Table 3.3). Previous studies have also indicated that environmental stresses such as temperature (Liu et al. 2011), water deficiency (Dai et al. 2009), nutrient supplementation (Ni et al. 2011), and light intensity (Li et al. 2009) affect the distribution of starch granules.

3.4.4. RVA analysis of refined flour and extracted starch

The thermal and pasting properties of starch are important functional properties, which are important in determining the behavior of different starches in various food matrices (Zaidul et al. 2007). Based on rheological principles, changes in the viscosity of starch is used to study the pasting properties of the same, and parameters such as extent of disintegration and degree of retrogradation can be determined using the pasting curve. In this study, RVA analysis was performed on refined wheat flour as well as isolated starch to determine the effect of glyphosate on pasting properties of wheat.

In the analysis of refined wheat flour pasting properties, glyphosate caused significant changes to all pasting properties except peak time and pasting temperature (Table 3.4). This could be due to interactions between starch and proteins, which influenced wheat flour pasting properties. Glyphosate’s effect on the fine chemistry of wheat starch and proteins may have
played a role. Cultivar effects were found to be significantly different for peak viscosity, hot paste viscosity, breakdown, setback, peak time, and pasting temperature. Location effects were significant for all RVA parameters except hot paste viscosity. The interaction between location x cultivar was significantly different for all parameters except peak time, and the cultivar x treatment interaction was not significantly different for any of the parameters determined. The location x treatment interaction was significant for all characteristics except peak time and pasting temperature, while the interaction between cultivar, location and treatment was not significant for any RVA parameters. Table 3.4 below summarizes this data and Figure 3.2 shows the pasting curves for the different glyphosate treatments.
Table 3.4. Effect of glyphosate was refined wheat flour pasting properties\(^a\)

<table>
<thead>
<tr>
<th>Effect</th>
<th>Peak viscosity (cP)</th>
<th>Hot paste viscosity (cP)</th>
<th>Breakdown (cP)</th>
<th>Final viscosity (cP)</th>
<th>Setback (cP)</th>
<th>Peak time (min)</th>
<th>Pasting temp (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment(^b)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2631.8 c</td>
<td>1544.6 b</td>
<td>1087.2 b</td>
<td>2648.7 c</td>
<td>1104.1 b</td>
<td>6.3 a</td>
<td>68.4 a</td>
</tr>
<tr>
<td>RP</td>
<td>2742.6 b</td>
<td>1618.7 a</td>
<td>1123.9 ab</td>
<td>2748.6 b</td>
<td>1129.8 b</td>
<td>6.4 a</td>
<td>68.3 a</td>
</tr>
<tr>
<td>SD</td>
<td>2805.7 a</td>
<td>1651.4 a</td>
<td>1154.2 a</td>
<td>2824.5 a</td>
<td>1173.1 a</td>
<td>6.3 a</td>
<td>68.6 a</td>
</tr>
<tr>
<td>Cultivar</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glenn</td>
<td>2827.2 a</td>
<td>1635.6 a</td>
<td>1191.6 a</td>
<td>2739.7 a</td>
<td>1104.1 b</td>
<td>6.4 a</td>
<td>68.5 a</td>
</tr>
<tr>
<td>Prosper</td>
<td>2626.2 b</td>
<td>1574.3 b</td>
<td>1051.9 b</td>
<td>2741.5 a</td>
<td>1167.2 a</td>
<td>6.3 b</td>
<td>68.4 a</td>
</tr>
<tr>
<td>Location</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carrington</td>
<td>2820.2 a</td>
<td>1628.6 a</td>
<td>1191.6 a</td>
<td>2834.9 a</td>
<td>1206.3 a</td>
<td>6.4 ab</td>
<td>68.4 b</td>
</tr>
<tr>
<td>Minot</td>
<td>2661.9 b</td>
<td>1612.1 a</td>
<td>1049.8 c</td>
<td>2653.8 b</td>
<td>1041.7 b</td>
<td>6.4 a</td>
<td>67.9 c</td>
</tr>
<tr>
<td>Prosper</td>
<td>2698.0 b</td>
<td>1574.1 a</td>
<td>1123.9 b</td>
<td>2733.1 b</td>
<td>1159.0 a</td>
<td>6.3 b</td>
<td>69.0 a</td>
</tr>
<tr>
<td>Loc x Trt(^c)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Car x Control</td>
<td>2724.8 b</td>
<td>1567.5 b</td>
<td>1157.3 a</td>
<td>2750.3 b</td>
<td>1182.8 a</td>
<td>6.3 a</td>
<td>68.5 a</td>
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<tr>
<td>Car x RP</td>
<td>2885.3 a</td>
<td>1674.7 a</td>
<td>1210.7 a</td>
<td>2889.2 a</td>
<td>1214.5 a</td>
<td>6.4 a</td>
<td>68.4 a</td>
</tr>
<tr>
<td>Car x SD</td>
<td>2850.5 a</td>
<td>1643.7 a</td>
<td>1206.8 a</td>
<td>2865.3 a</td>
<td>1221.7 a</td>
<td>6.3 a</td>
<td>68.4 a</td>
</tr>
<tr>
<td>Minot x Control</td>
<td>2655.5 a</td>
<td>1604.2 a</td>
<td>1051.3 a</td>
<td>2652.5 a</td>
<td>1048.3 a</td>
<td>6.4 a</td>
<td>67.8 a</td>
</tr>
<tr>
<td>Minot x RP</td>
<td>2699.3 a</td>
<td>1642.0 a</td>
<td>1057.3 a</td>
<td>2693.2 a</td>
<td>1051.2 a</td>
<td>6.4 a</td>
<td>67.9 a</td>
</tr>
<tr>
<td>Minot x SD</td>
<td>2630.8 a</td>
<td>1590.2 a</td>
<td>1040.7 a</td>
<td>2615.7 a</td>
<td>1025.5 a</td>
<td>6.4 a</td>
<td>68.0 a</td>
</tr>
<tr>
<td>Pros x Control</td>
<td>2515.2 c</td>
<td>1462.2 c</td>
<td>1053.0 b</td>
<td>2543.3 c</td>
<td>1081.2 b</td>
<td>6.3 a</td>
<td>69.0 a</td>
</tr>
<tr>
<td>Pros x RP</td>
<td>2643.2 b</td>
<td>1539.5 b</td>
<td>1103.7 b</td>
<td>2663.3 b</td>
<td>1123.8 b</td>
<td>6.3 a</td>
<td>68.7 ab</td>
</tr>
<tr>
<td>Pros x SD</td>
<td>2935.7 a</td>
<td>1720.5 a</td>
<td>1215.2 b</td>
<td>2992.5 a</td>
<td>1272.0 a</td>
<td>6.3 a</td>
<td>69.2 a</td>
</tr>
</tbody>
</table>

\(^a\) LSD for each location is given at \(P \leq 0.05\) significance level, where values followed by the same letter are not significantly different at \(P \leq 0.05\)

\(^b\) RP: Ripe treatment, SD: soft dough treatment

\(^c\) Loc: location, Car: Carrington, Pros: Prosper, LSD for each location is given at \(P \leq 0.05\) significance level, where values followed by the same letter are not significantly different at \(P \leq 0.05\)
As mentioned previously, RVA analysis was also performed on isolated wheat starch to determine if the effects on starch pasting properties is caused by physicochemical properties of starch alone, or if starch-protein interactions play a role in the observed results. In the starch RVA analysis significant differences between treatments were not found for any of the pasting characteristics analyzed (Table 3.5). Significant differences were found between cultivars for peak viscosity, breakdown, final viscosity, set back and peak time. Significant differences between locations was observed for hot paste viscosity, breakdown and setback, whereas the interactions were also non-significant except for the location x cultivar interaction for peak viscosity, hot paste viscosity and final viscosity, while location x treatment was significant for pasting temperature and set back.
Table 3.5. Effect of glyphosate on wheat starch pasting properties\textsuperscript{a}

<table>
<thead>
<tr>
<th>Effect</th>
<th>Peak viscosity (cP)</th>
<th>Hot paste viscosity (cP)</th>
<th>Breakdown viscosity (cP)</th>
<th>Final viscosity (cP)</th>
<th>Setback viscosity (cP)</th>
<th>Peak time (min)</th>
<th>Pasting temperature (℃)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment\textsuperscript{b}</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2779.1 a</td>
<td>2109.6 a</td>
<td>669.5 a</td>
<td>3464.1 a</td>
<td>1354.6 a</td>
<td>7.0 a</td>
<td>89.0 a</td>
</tr>
<tr>
<td>RP</td>
<td>2760.2 a</td>
<td>2097.6 a</td>
<td>662.6 a</td>
<td>3444.4 a</td>
<td>1346.8 a</td>
<td>7.0 a</td>
<td>89.0 a</td>
</tr>
<tr>
<td>SD</td>
<td>2800.4 a</td>
<td>2117.2 a</td>
<td>683.2 a</td>
<td>3511.8 a</td>
<td>1394.6 a</td>
<td>7.0 a</td>
<td>88.9 a</td>
</tr>
<tr>
<td>Cultivar</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glenn</td>
<td>2918.3 a</td>
<td>2104.8 a</td>
<td>813.6 a</td>
<td>3621.8 a</td>
<td>1517.0 a</td>
<td>7.0 a</td>
<td>88.8 a</td>
</tr>
<tr>
<td>Prosper</td>
<td>2641.4 b</td>
<td>2111.4 a</td>
<td>530.0 b</td>
<td>3325.1 b</td>
<td>1213.6 b</td>
<td>7.0 b</td>
<td>89.0 a</td>
</tr>
<tr>
<td>Location</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carrington</td>
<td>2716.8 a</td>
<td>2035.1 b</td>
<td>681.7 a</td>
<td>3367.2 a</td>
<td>1332.2 b</td>
<td>7.0 a</td>
<td>89.2 a</td>
</tr>
<tr>
<td>Minot</td>
<td>2794.7 a</td>
<td>2181.4 a</td>
<td>613.3 b</td>
<td>3454.5 a</td>
<td>1273.1 b</td>
<td>7.0 a</td>
<td>88.3 a</td>
</tr>
<tr>
<td>Prosper</td>
<td>2828.2 a</td>
<td>2107.9 ab</td>
<td>720.3 a</td>
<td>3598.6 a</td>
<td>1490.7 a</td>
<td>7.0 a</td>
<td>89.2 a</td>
</tr>
</tbody>
</table>

\textsuperscript{a} LSD for each location is given at $P \leq 0.05$ significance level, where values followed by the same letter are not significantly different at $P \leq 0.05$

\textsuperscript{b} RP: Ripe treatment, SD: soft dough treatment

The role and importance of wheat and cereal starch properties in food systems have been investigated by many researchers over the years. For example, Shibanuma et al. (1996) determined that high molecular weight amylose and amyllopectin as well as low amounts of extra-long chains of amyllopectin increase the viscosity of wheat starches during pasting, and Fitzgerald et al. (2009) concluded that amylose content and amyllopectin fine structure of rice starch are important characteristics that determine grain quality, flour quality and pasting properties. Zhang et al. (2017) studied the effect of soil drought conditions on the physicochemical properties of wheat starch and reported that moderate soil drought conditions lead to increases in starch accumulation, proportion of large starch granules, content of amylose and amyllopectin long-branch chain contents and amyllopectin branch length. Additionally, these conditions caused decreases in gelatinization enthalpy, higher gelatinization temperature, retrogradation enthalpy and retrogradation percentage. The opposite results were found for severe soil drought conditions. Thus, the significant differences that were found between
locations could be due to different environmental conditions leading to differences in the fine chemistry of starch. Kaur et al. (2016) studied the physicochemical properties of Indian durum wheat cultivars and determined that cultivars with higher proportions of large granules show increased peak viscosity, breakdown viscosity (analyzed using rheometer) in contrast to cultivars with higher proportion of small granules. In the current study, the glyphosate treated samples had higher proportion of A-type granules, and higher values for peak viscosity and breakdown viscosity similar to the observation of Kaur et al. (2016). Thus, the differences observed with regard to flour pasting properties could be associated with the differences in starch granule distribution described in the previous section, and also with the fine chemistry of amylopectin and interactions between starch and other grain components. The results also indicate that interactions between starch other grain components such as protein are important in determining flour pasting properties because glyphosate treatment did not cause significant differences in starch pasting properties. Thus, glyphosate can affect wheat flour pasting properties when starch is in contact with prominent grain components such as proteins, and not when starch is in isolation. This is also an indication that glyphosate may also be causing changes to the chemistry of gluten proteins.

3.4.5. **Protein composition and molecular weight analysis using SE-HPLC techniques**

Wheat gluten proteins, composed of gliadins and glutenins, are a type of storage proteins, which are very important in dough functionality (Shewry 2009). The role of these proteins in dough functionality has been extensively discussed in literature. For example, Malalgoda et al. (2018) studied historical and modern hard red spring wheat cultivars and determined that glutenins and ω-gliadin proteins are important for baking quality. Additionally, Ohm et al. (2009) and Ohm et al. (2010) determined that SDS insoluble glutenin proteins show positive
correlations with dough quality characteristics, whereas SDS soluble proteins show negative correlations with the same characteristics. The role of gliadin proteins in dough and baking quality is still under debate where some studies show that gliadin proteins have a negative effect (Fido et al. 1997; Khatkar et al. 2002; Uthayakumaran et al. 2001), whereas others have shown that certain gliadin fractions have positive effects (Huebner and Bietz 1986; Park et al. 2006). In this study, SE-HPLC was used to analyze the effects of glyphosate on the composition of gluten proteins (Figure 3.3).

![Figure 3.3. SE-HPLC profiles of SDS extractable and unextractable proteins](image_url)

In this study, fractions for both extractable and unextractable proteins were determined according to the following retention times. F1: 3.5-5.2, F2: 5.2-5.6, F3: 5.6-6.3 and F4: 6.3-7.7 min. Table 3.6 shows the effect of glyphosate application time on the proportion of different fractions of extractable and unextractable proteins.
Table 3.6. Effect of glyphosate timing on SDS extractable and unextractable proteins\(^a\)

<table>
<thead>
<tr>
<th>Effect</th>
<th>SDS extractable proteins</th>
<th>SDS unextractable proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F1</td>
<td>F2</td>
</tr>
<tr>
<td>Treatment(^b)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>17.64 a</td>
<td>7.71 a</td>
</tr>
<tr>
<td>RP</td>
<td>17.19 a</td>
<td>7.69 a</td>
</tr>
<tr>
<td>SD</td>
<td>17.48 a</td>
<td>7.50 a</td>
</tr>
<tr>
<td>Cultivar</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glenn</td>
<td>16.40 b</td>
<td>7.72 a</td>
</tr>
<tr>
<td>Prosper</td>
<td>18.47 a</td>
<td>7.54 a</td>
</tr>
<tr>
<td>Location</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carrington</td>
<td>17.77 a</td>
<td>7.51 a</td>
</tr>
<tr>
<td>Minot</td>
<td>18.25 a</td>
<td>7.94 a</td>
</tr>
<tr>
<td>Prosper</td>
<td>16.30 a</td>
<td>7.45 a</td>
</tr>
</tbody>
</table>

\(^a\) LSD for each location is given at \(P \leq 0.05\) significance level, where values followed by the same letter are not significantly different at \(P \leq 0.05\)

\(^b\) RP: Ripe treatment, SD: soft dough treatment

As demonstrated in Table 3.6, glyphosate treatment did not have a significant effect on the proportion of the different SDS extractable and unextractable protein fractions, although the chemical characteristics of proteins could be affected. When glyphosate is applied, the deposition of proteins may be affected, but since albumin and globulin proteins are deposited early during protein biosynthesis, glyphosate may only cause minor changes in the amount of gliadins and glutenins. Thus, overall the treatment may not cause significant changes in protein composition. However, cultivar and location had significant effects on the proportion of some of the protein fractions. Additionally, interactions between the main effects did not show significant differences for most of the protein fractions. Previous work on wheat gluten proteins have also shown that genotype, environment and genotype x environment can have significant effects on the amount of different gluten protein fractions. For example, Payne (1987) studied the genetics of wheat gluten proteins and how allelic variation can contribute to variations in breadmaking quality. Furthermore, Panozzo and Eagles (2000) also studied how genetics, environmental
conditions and the interaction between these factors is important in the chemistry and functionality of wheat gluten proteins.

### 3.4.6. Shikimic acid quantitation in whole wheat flour

As explained in previously, glyphosate inhibits the enolpyruvylshikimate phosphate synthase (EPSPS) enzyme, which disrupts the shikimic acid pathway leading to the plant being deprived of aromatic amino acids such as tyrosine, tryptophan and phenylalanine which are products of this pathway (Williams et al. 2000). Shikimic acid/ shikimate accumulates as a result of the interaction between glyphosate and EPSPS because shikimic acid is found earlier in the shikimic acid pathway and is a precursor for aromatic amino acids. In wheat, application of glyphosate has been found to significantly increase the shikimic acid content (Bresnahan et al. 2003). In this study, shikimic acid was quantified using HPLC techniques, and the figure below summarizes the results for the three glyphosate treatments.

![Figure 3.4. Accumulation of shikimic acid after glyphosate treatment across all the cultivars and locations](image)

Glyphosate application at both soft dough and ripe stages significantly increased shikimic acid accumulation in this study. As shown in Figure 3.4, the highest amount of shikimic acid was found for the soft dough treated samples, whereas ripe stage treatment was the next, and the
lowest amount of shikimic acid was found in the control. Therefore, as the results show the
disruption of the shikimic acid pathway by glyphosate induces the accumulation of shikimic
acid, and the amount of accumulated shikimic acid is higher when glyphosate is applied
relatively early during physiological maturation. Similar observations were made by Bresnahan
et al. (2003). In addition to treatment, the amount of shikimic acid was significantly different
between cultivars and locations and was significant for the two-way and three-way interactions.
Thus, shikimic acid accumulation is affected by multiple factors. Some studies have implied that
the accumulation of shikimic acid could be a contributing reason for plant deterioration after the
application of glyphosate (Henry et al. 2007). The results of the current study can be used to
support this view, however further research is needed to understand the effects of shikimic acid
accumulation on the physicochemical properties of affected plants.

3.5. Conclusions

In this study, the effect of glyphosate on wheat starch physicochemical properties, protein
composition and shikimic acid accumulation was determined. Two wheat cultivars were grown
at three locations and glyphosate was sprayed at soft dough and ripe stages to analyze the effect
of glyphosate application time on these properties. Glyphosate treatment did not change the
percentage or the molecular weight of amyllose and amylopectin. However, glyphosate treatment
affected the distribution of A and B type starch granules, where B-type granules decreased in
proportion and A-type granules increased. As per starch digestibility characteristics, glyphosate
treatment at ripe stage increased the percentage of RDS and vice versa for SDS. Glyphosate
treatment also decreased the percentage of RS. The pasting properties of wheat flour were also
impacted by glyphosate where, RVA peak viscosity and final viscosity were significantly higher
at soft dough stage application. However, starch RVA analysis did not show significant
differences for treatment indicating that changes in flour pasting properties could be associated
with changes glyphosate may be causing in gluten protein chemistry. Moreover, glyphosate did
not affect gluten protein composition as demonstrated by the SE-HPLC analysis. As for shikimic
acid analysis, the results show that accumulation is highest at soft dough treatment and lower for
ripe treatment as well as the control. Overall, these results show that glyphosate application can
affect wheat starch physicochemical characteristics such as granule size distribution, digestibility
and pasting properties as well as the shikimic acid accumulation. The changes in the
physicochemical properties of starch could be caused by the effect of glyphosate in the
deposition of starch granules. When glyphosate is applied, the deposition of B-type granules is
interrupted leading to comparably higher proportion of A-type granules as presented in this
study. Additionally, structural changes to starch molecules caused by effects on photosynthesis,
carbon flow and/or enzyme activity through the metal chelation ability of glyphosate could lead
to changes in digestibility. These changes together with changes caused to the chemical
properties of gluten proteins could be responsible for the changes in flour pasting properties.
However, the cause for the observations is not fully understood. As the results show, the
inhibition of the shikimic acid pathway directly causes a spike in shikimic acid levels, and as
demonstrated in this study, the resulting interference in the carbon flow and other biochemical
pathways could be associated with many of the changes seen in starch physicochemical
properties. Overall, this study shows that further investigations focused on understanding the
effect of glyphosate on plant biochemical pathways are needed to firmly establish the association
between glyphosate’s mode of action and plant biosynthetic pathways.
3.6. References


CHAPTER 4. EFFECT OF GLYPHOSATE ON THE FINE CHEMISTRY OF SPRING WHEAT PROTEINS

4.1. Abstract

When glyphosate is used as a pre-harvest aid, the main purpose is enabling timely harvest. Glyphosate inhibits a key enzyme in the shikimic acid pathway, thereby it disrupts the biosynthesis of aromatic amino acids. During wheat cultivation, glyphosate is used as a pre-harvest desiccant. It is recommended to be applied at least seven days prior to harvest during the ripe stage of physiological maturity when the moisture content is 30% or less. At times, some grain may not be at this moisture content due to non-uniform maturation. In this context, the goal of this study was to determine the effect of glyphosate of the chemistry of wheat gluten proteins. For this purpose, a greenhouse study was conducted where glyphosate was sprayed at soft dough and ripe stage to two distinct groups of the wheat cultivar ‘Glenn’. For the control samples, water was sprayed instead of glyphosate at the same developmental stages. Samples were collected prior to spraying, a day after spraying and every three days until harvest. Whole wheat flour samples were then analyzed for amino acid composition, secondary protein structure, gluten protein composition and molecular weight and shikimic acid accumulation. The results of the study indicated that pre-harvest application of glyphosate does not impact the amino acid composition, protein secondary structure and gluten protein composition. However, glyphosate application decreased the molecular weight, SDS extractable and unextractable proteins, and significantly increased the amount of shikimic acid accumulation, specifically when applied at soft dough stage. Thus, this study indicates that glyphosate does not change the primary and secondary structure of proteins, although pre-harvest glyphosate application can cause significant
differences in the molecular weight and therefor may change the physicochemical properties of wheat proteins, while significantly increasing the shikimic acid content in affected plants.

4.2. Introduction

Wheat gluten proteins are found in endosperm of the wheat kernel and are considered as storage proteins, whereas proteins such as albumins and globulins are found in the germ (Bonomi et al. 2013). The solid gluten mass is comprised of 75-85% proteins and 5-10% lipids and is responsible for the visco-elastic properties in wheat dough (Wieser 2007). Thus, one of the key characteristics of wheat, which is its ability to form dough is primarily due to the physical and chemical properties of gluten proteins (Shewry 2009). Gluten-forming proteins, which are involved in the dough formation process can be categorized into two groups, namely glutenins and gliadins (Delcour and Hoseney 2010). Bonomi et al. (2013) reported that the molecular weight of gliadin is between 28,000 and 55,000 and that these proteins are monomeric. Gliadin proteins can be subdivided into four groups according to their mobility of low pH electrophoresis, which are α, β, γ and ω (Wieser 2007). Glutenin proteins are polymeric and are held together via disulfide bridges. These proteins have a molecular weight range between 500,000 to more than 10 million. As Shewry et al. (1986) reported, the genes encoding gluten proteins have resulted from events such as duplication and translocation, which has resulted in the insertions and deletions of peptides sequences as well as amino acid substitutions.

Woychik et al. (1961) determined that wheat gluten proteins contain very high levels of glutamic acid and proline. Considering the overall amino acid composition, glutamic acid accounts for approximately 35- 40% and proline accounts for 13 to 23%. Thus, these amino acids constitute approximately half of the total amino acid composition of wheat gluten proteins. Additionally, Woychik et al. (1961) found that the levels of glutamic acid and proline are
consistently high in different fractions of gluten proteins such as gliadin proteins and water-soluble gluten proteins, with the highest levels detected in gliadins and the least in glutenin proteins. Rombouts et al. (2009) used high performance anion exchange chromatography to determine the amino acid composition of wheat gluten proteins and made similar observations, where they found that these proteins contain high levels of glutamine and glutamic acid as well as proline. Shewry (2009) studied the amount of essential amino acids in wheat proteins and reported that wheat proteins have high content of leucine, phenylalanine and tyrosine, and low amounts of lysine compared to the recommended levels of essential amino acids.

The secondary structure of wheat gluten proteins has also been investigated using Fourier-transform infrared (FTIR) spectroscopy techniques as described by Bock and Damodaran (2013). Using such methods the percentage of secondary structures such as β-sheets, β-turns, α-helices and random coils can be detected as previously examined by Marti et al. (2016), who reported that hard wheat flour contains approximately 42% β-sheets, 32% β-turn, 10% α-helices and 12% random coils. Factors such as addition of bran (Bock and Damodaran 2013), polysaccharides (Nawrocka et al. 2018), application of electric fields (Singh et al. 2016) and silver nanoparticles (for antimicrobial properties) (Nawrocka 2014) have shown to affect the secondary structure of wheat gluten proteins.

Glyphosate is the most widely used herbicide in the world and its application has increased rapidly in the last few decades due to reasons such as the introduction of glyphosate-resistant crops and agricultural practices such as the pre-harvest application of herbicides for desiccation (Benbrook 2016). Glyphosate’s mode of action is highly specific to higher plants as it targets the shikimic acid pathway which is highly conserved in such plants (Williams et al. 2000). Glyphosate inhibits the enolpyruvylshikimate phosphate synthase enzyme in the shikimic
acid pathway which catalyzes the reaction which converts shikimate/shikimic acid to 3-enolpyruvylshikimate-5-phosphate, which is a precursor to chorismate, which gives rise to aromatic amino acids and secondary metabolites (Williams et al. 2000). Previous studies on glyphosate’s effect on plant biochemistry have shown that glyphosate can affect the carbon flow (Zobiole et al. 2011) and accumulation of shikimic acid in affected crops Bresnahan et al. (2003). Although there have not been many studies investigating the effect of glyphosate on the biochemical properties of wheat proteins, Faheed (2012) reported that herbicides can have negative effects on the protein content and proline levels of wheat proteins. Additionally, Alla et al. (2008) found that early application of herbicides such as metribuzin, butachlor and chlorimuron-ethyl slightly reduced the activity of nitrate reductase and nitrite reductase, whereas activity of enzymes such as glutamine synthetase and glutamate synthase are greatly reduced. Wang and Zhou (2006) studied the effect of chlorimuron-ethyl on wheat physiological mechanisms and determined that this herbicide can damage the antioxidative defense systems in wheat and cause dose dependent changes in the soluble protein content in roots. However, the effect of different herbicides could be determined by the mode of action of the respective herbicides.

Glyphosate can affect the deposition and the biochemistry of wheat gluten protein as it disrupts the biosynthesis of aromatic amino acids. Additionally, glyphosate could affect the composition of gluten proteins as it could interfere to different degrees the deposition of gliadin and glutenin proteins. These aspects of glyphosate use as a pre-harvest desiccant during wheat cultivation has not been studied extensively. In this context, the aim of the study was to examine the effect of glyphosate timing on wheat gluten protein composition and the chemistry of these proteins.
4.3. Materials and methods

4.3.1. Design of greenhouse study

A randomized complete block design was used for this experiment which was designed to determine the effect of glyphosate on the fine chemistry of spring wheat proteins. There were four different treatments in the experiment, where glyphosate was sprayed at soft dough stage, ripe stage and where water was sprayed instead of glyphosate at the same developmental stages (controls). A single seed of cultivar Glenn was placed in each of the cones filled with soil (PRO-MIX® LP15, Premier Tech Horticulture, Quakertown, PA). Multicote™ (Haifa Group, Altamonte Springs, FL) was applied (1/8th of a teaspoon) 2 weeks after planting at the three-leaf stage. When soft dough stage was used, glyphosate was sprayed in a spray booth (Generation III Research Sprayer, Devries Manufacturing, Hollandale, MN) at a rate of 1.1 kg of active ingredient/ha, and the control was sprayed with water. The plants were taken back into the greenhouse room approximately 4 hours after spraying. Heads were collected before spraying, a day after spraying and every three days until harvest (physiological maturity). The same procedure was performed when ripe stage of physiological maturity was reached. The heads were dried at 30°C in a forced air oven (Shel Lab, Sheldon Manufacturing, Inc. Cornelius, OR) for two days, then cleaned using a wheat head thresher (Precision Machine Co. Inc., Lincoln, NE). The kernels were then milled using a ball mill (Retsch Mixer Mill MM400, Retsch, Haan, Germany) at a frequency of 30/s for 2 min. The samples were then stored at -20°C until analysis.

4.3.2. Amino acid analysis

The complete amino acid profiles of the harvest samples obtained from the greenhouse. For this purpose, Association of Analytical Communities (AOAC) Official Method 982.30 E (a, b, c), chp. 45. 3. 05, 2006. In short, two aliquots of each sample were subjected to acid
hydrolysis, acid oxidation, which was followed by acid hydrolysis again and alkaline hydrolysis. The final hydrolysate was used to determine the amino acid composition using an amino acid analyzer. The percentage of each amino acid was calculated based on the nitrogen content specific to that amino acid.

4.3.3. **FTIR analysis of dough samples**

The harvest samples from the greenhouse study were used in the FTIR analysis of dough samples and used to determine the secondary structure of proteins. This was performed according to a method previously described by Quayson et al. (2016). A farinograph-AT (C. W. Brabender, Hackesack, NJ) device with a 10g mixing bowl was used to determine optimal water absorption, which is the amount of water required to reach the 500 BU line. The temperature of the mixing bowl was kept at 30°C using a temperature-controlled water bath and the water added to reach the desired consistency was also kept at 30°C. For FTIR analysis of dough, the flour samples were mixed until peak time/dough development time and removed from the mixing bowl with minimal manipulation. During this experiment, dough samples were collected in duplicate.

An attenuated total reflectance (ATR) Fourier transform infrared (FTIR) spectrophotometer (Bruker Tensor 37, Bruker Optics, Billerica, MA) was used to determine the conformation of proteins in the dough samples. The instrument was equipped with a horizontal multi-reflectance zinc selenide (ZnSe) crystal accessory. Spectra were collected at room temperature in the wave length range of 600 – 4000 cm\(^{-1}\), with each spectrum having 32 scans at 4 cm\(^{-1}\) resolution. Prior to analyzing each sample, a background spectrum of the empty trough sampling plate was collected. The dough samples were analyzed within 10 min of collecting at farinograph peak time and a minimum of 4 spectra were used for the analysis of protein
secondary structure. To prepare the sample for analysis, the dough sample was firmly pressed against the crystal to remove air and to maximize contact. A method previous described by Bock and Damodaran (2013) was used to analyze the samples using the OPUS software version 7.0 (Bruker Optics). During data analysis, reference H$_2$O-D$_2$O mixtures that matched the moisture content of the dough samples (43 – 48%) were obtained and vector normalized using the software and used for the subtraction of water contribution in the amide I region 1600 - 1700 cm$^{-1}$ of the normalized spectra. To quantitate the secondary protein structures in the amide I region the second-derivative spectra using a five-point Sayitzky-Golay function was used as described by Bock and Damodaran (2013). The spectral regions for β-sheets, unordered, α-helix and β-turn were 1620-1644, 1644-1652, 1652-1660 and 1660-1685 respectively. To determine the percentage of each secondary structure, the second-derivative area corresponding to each of the structures was calculated as a percentage of the total area of the amide I region.

4.3.4. **FTIR analysis of flour samples**

The infrared spectra of flour samples were determined according to the method of Marti et al. (2016) using an ATR-FTIR spectrophotometer (Thermoscientific Nicolet is10, smart ITX). Background spectra were collected prior to analyzing each sample. A wave length range of 4000-600 cm$^{-1}$ with an average of 32 scans at 4 cm$^{-1}$ resolution were used.

4.3.5. **Size exclusion HPLC analysis of gluten proteins**

SDS extractable and unextractable proteins were extracted according to the method of Gupta et al. (1993) with modifications as per Ohm et al. (2009). The extraction buffer used in the extraction procedure comprised of 0.5% SDS and 0.05 M sodium phosphate, pH 6.9. To obtain the SDS extractable proteins, 10 mg of sample (adjusted to 14% moisture content) was weighed out to which 1 mL of extraction solution was added and stirred for 5 min using a vortex mixer at
2500 rpm (Fischer Scientific, Waltham, MA, U.S.A.). Afterwards the samples were centrifuged at 20,000 rpm (Eppendorf Centrifuge 5424) for 15 min, then the supernatant was filtered through a 0.45 µm PVDF membrane (Sun Sri, Rockwood, TN, U.S.A.). To inactivate enzymes and prevent degradation, the SDS extractable protein samples were finally heated at 80°C for 2 min in a water bath. The SDS unextractable proteins was obtained from the pellet of the centrifugation step mentioned above, to which 1 mL of the same extraction buffer was added and sonicated in a probe type sonicator (Sonic Dismembrator 100, Fisher Scientific, Waltham, MA, U.S.A.) for 30 seconds at 10W power setting. Following sonication, centrifugation, filtration and heating were performed as explained for the SDS extractable proteins. An Agilent 1100 series narrow bore size exclusion column (300 x 4.5 mm, BIOSEP SEC S4000, Phenomenex, Torrance, CA, U.S.A.) and a guard cartridge (BIOSEP SEC S4000, Phenomenex) together with an Agilent 1200 photodiode array detector (Agilent Technologies, Waldbronn, Germany) at 214 nm were used to analyze the SDS extractable and unextractable proteins. The mobile phase used during the analysis was 50% acetonitrile in water with 0.1% (v/v) trifluoroacetic acid. The flow rate was 0.5 min/min and the injection volume was 10 µL. Molecular weight analysis of SDS extractable and unextractable fractions was performed as previously described by Bean and Lookhart (2001).

As previously described in Ohm et al. (2009) and Malalgoda et al. (2018) the MATLAB software (2015, The MathWorks, Natick, MA, U.S.A.) and an in-house program was used to process the data. The total area for SE-HPLC profiles for SDS-extractable and unextractable proteins were used to determine quantitative and qualitative changes in gluten protein of spring wheat samples used in the study.
4.3.6. Quantitation of shikimic acid

The shikimic acid extraction method of Zelaya et al. (2011) was used in the current study. As the starting material, 0.2 g of whole wheat flour was weighed out to which 2 mL of 0.25 N HCl was added and pulversized for five min using a homogenizer system (SCIENCEWARE® Wilmad LabGlass Micro Tube Homogenizer System, Bel-Art, VWR International, Radnor, PA. U.S.A.). The extractions were then subjected to sonication for 1 hour in an ultrasonic sonicator (Branson 1200m Bransonics Ultrasonic Cleaner, Branson Ultrasoundics Coorporation, Danbury, CT, U.S.A) after which the samples were centrifuged for 15 min at 10,000 g (Allegra 64R Centrifuge, Backman Coulter). Finally, the extracts (supernatants) were filtered through a 0.45 µm nylon membrane filter (VWR International, Radnor, PA, U.S.A).

The method for HPLC analysis was based on the method reported by Feng et al. (2004) where the quantitation was based on standard curves produced using analytical grade shikimic acid (10 - 200 ppm). The zero level was 0.25N HCl in this analysis. The column used for the analysis was a Phenomenex Luna 5µm C18 column (250 x 4.6 mm) (Phenomenex, Torrance, CA, U.S.A) and an Agilent 1100 variable wave length detector (Agilent Technologies) was used to detect the signal at 215 nm. Two solvents (A and B) were used during this experiment, where Solvent A comprised on 6 mM phosphoric acid, and solvent B was 100% methanol. The flow rate during the analysis was 1 mL/min and the injection volume was 20 µL. The following linear gradient was used during the analysis, 0 – 6 min: 0% B, 6 – 10 min: % B was increased to 100%, 10 – 20 min: % B was maintained at 100%, 20-21 min: % B was reduced to 0% and 21-30 min: 0% B. The ChemStation software was used to quantify the amount of shikimic acid in the samples.
4.3.7. Statistical analysis

The SAS software (Version 9.3, SAS Institute, Cary, NC, USA) was used to perform the statistical analysis (ANOVA) and mean separation (Fisher’s protected LSD) of the obtained data.

4.4. Results and discussion

4.4.1. Amino acid composition of harvest samples

Glyphosate inhibits the shikimic acid pathway in plants, thereby stops the production of aromatic amino acids such as, phenylalanine, tyrosine and tryptophan. In this study, the amino acids composition of the harvest samples was analyzed to determine changes in amino acid composition caused by glyphosate application at soft dough and ripe stages. Table 4.1 shows the results of this analysis.

As indicated in Table 4.1, significant differences were detected in the amino acid composition of whole wheat flour samples. In general, the results were in a very narrow range raising the possibility that although statistically significant, the amino acid composition may not be different physiologically, and may not result in differences in wheat biochemistry or physiological functions.
As mentioned previously, glyphosate application inhibits the shikimic acid pathway, which is responsible for the production of aromatic amino acids such as tyrosine, tryptophan and phenylalanine. Therefore, we expected to observe substantial differences in the amount of these amino acids. Tyrosine content was not significantly lower in the treated samples compared to the control (Table 4.1). As for phenylalanine content, the ripe control showed the highest content and the lowest was found in the soft dough control. The treated samples were significantly

### Table 4.1. Amino acid composition of harvest samples

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Glyphosate Content of amino acid (W/W% g per 100 g sample)</th>
<th>Control Content of amino acid (W/W% g per 100 g sample)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Soft dough</td>
<td>Ripe</td>
</tr>
<tr>
<td>Taurine §</td>
<td>0.17 a</td>
<td>0.17 a</td>
</tr>
<tr>
<td>Aspartic Acid</td>
<td>0.80 ab</td>
<td>0.78 b</td>
</tr>
<tr>
<td>Threonine</td>
<td>0.48 a</td>
<td>0.47 ab</td>
</tr>
<tr>
<td>Serine</td>
<td>0.71 ab</td>
<td>0.70 ab</td>
</tr>
<tr>
<td>Glutamic Acid</td>
<td>5.68 b</td>
<td>5.56 b</td>
</tr>
<tr>
<td>Proline</td>
<td>1.84 b</td>
<td>1.80 b</td>
</tr>
<tr>
<td>Lanthionine §</td>
<td>0.02 a</td>
<td>0.01 a</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.73 ab</td>
<td>0.71 b</td>
</tr>
<tr>
<td>Alanine</td>
<td>0.59 ab</td>
<td>0.57 b</td>
</tr>
<tr>
<td>Cysteine</td>
<td>0.40 a</td>
<td>0.39 ab</td>
</tr>
<tr>
<td>Valine</td>
<td>0.77 a</td>
<td>0.75 b</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.28 a</td>
<td>0.28 a</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0.66 b</td>
<td>0.64 c</td>
</tr>
<tr>
<td>Leucine</td>
<td>1.20 ab</td>
<td>1.17 b</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.48 a</td>
<td>0.48 a</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.88 b</td>
<td>0.86 b</td>
</tr>
<tr>
<td>Hydroxylysine</td>
<td>0.03 a</td>
<td>0.03 a</td>
</tr>
<tr>
<td>Ornithine §</td>
<td>0.01 a</td>
<td>0.01 a</td>
</tr>
<tr>
<td>Lysine</td>
<td>0.47 a</td>
<td>0.46 b</td>
</tr>
<tr>
<td>Histidine</td>
<td>0.42 ab</td>
<td>0.41 b</td>
</tr>
<tr>
<td>Arginine</td>
<td>0.79 a</td>
<td>0.77 ab</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>0.19 a</td>
<td>0.18 ab</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>17.60 ab</strong></td>
<td><strong>17.19 b</strong></td>
</tr>
</tbody>
</table>

*Results are expressed on an as is basis; § indicates non-proteinogenic amino acids; Values followed by the same letter in each row are not significantly different at $P \leq 0.05$ significance level.*
different than the two control samples. The range for phenylalanine was between 0.83 and 0.91%. The highest amount of tryptophan was observed in the ripe treated samples, and the rest of the samples showed the same value. The range for tyrosine was between 0.18 and 0.19%, indicating that in a physiological context, the observed differences may not cause changes to the chemical composition in its entirety. For most of the other amino acids quantified, the highest values was found in the control ripe sample, whereas the lowest was in the control soft dough sample, and treated samples showed intermediary values. Thus, overall these results suggest that glyphosate application may not lead to lower levels of aromatic amino acids, and that pre-harvest application of glyphosate does not appear to significantly alter the amino acid composition of wheat proteins. This maybe because amino acids for storage protein biosynthesis comes from the breakdown of proteins during the vegetative state, making the effect glyphosate insignificant.

The obtained results are comparable to that of Li et al. (2006) who studied the amino acid composition of whole wheat flour. Abdel-Aal and Hucl (2002) studied the amino acid composition and protein digestibility of different wheat varieties and determined these parameters for Katepwa hard red spring wheat. According to their results, tyrosine, tryptophan and phenylalanine were found at 2.6, 1.4 and 4.8% respectively. Thus, the values obtained in the present study are somewhat lower than that of Abdel-Aal and Hucl (2002). The same trend was seen for other amino acids as well. Similar observations were made when comparing these results with that of Yang et al. (2012), who studied Korean wheat cultivars. As Prabhasankar and Rao (2001) determined, milling methods have an influence on the protein and lipid composition of whole wheat flour, further adding to the difficulty of comparison. Additionally, Wang (2001), who studied the impact of glyphosate on purple nutsedge reported that shikimic acid accumulation could be the primary reason for glyphosate’s toxicity. In this study, only
tryptophan showed significant differences in the treated samples, whereas other amino acids did not show any differences. In this context, glyphosate’s toxicity towards wheat could also be due to shikimic acid accumulation and not due to deficiencies or changes in amino acid composition. As previous work highlighted, the effect of herbicides on amino acid composition varies widely, where Nema Alla et al. (2008) found that herbicides such as metribuzin, butachlor and chlorimuron-ethyl elevated the levels of aliphatic, aromatic and total amino acids in wheat and maize. Moreover, various abiotic stresses and crop management systems could also have an impact on the amino acid composition of cereals (Byers and Bolton 1979; Halford et al. 2015; Sosulski et al. 1963).

### 4.4.2. Protein secondary structure analysis of harvest samples.

Gluten structure is a dynamic entity (Wellner et al. 2005). The structural features of gluten affects protein interactions that take place during processing events such as, mixing, proofing and baking (Bock et al. 2013). Moreover, these secondary structures affect the formation and characteristics of the gluten network in dough and baked food products. Thus, the structural analysis of gluten proteins can be considered as an indicator of gluten quality. Fourier transform infrared spectroscopy (FTIR) has been a widely used technique for the analysis of wheat gluten protein secondary structures in the last few decades (Georget and Belton 2006). In most cases, four structural conformations, which include β-turn, β-sheet, α-helix and random coil are reported. High molecular weight glutenin subunits have been found be a determining factor regarding the viscoelastic properties of wheat dough (Payne 1987). These proteins, in solution form a loose spiral structure based on β-reverse turns, which are elastic, are flanked by non-repetitive domains which are dominated by α-helix structures (Shewry et al. 2002). Studies have shown that processing of gluten leads to the conversion of β-turn to β-sheets (Wellner et al.)
This technique has been instrumental in understanding the molecular basis of viscoelastic properties observed in the wheat gluten network (Bruun et al. 2007). For example, the effect of hydration on the secondary structure of gluten proteins revealed that \( \beta \)-sheet increased as a response to increased hydration, suggesting that interactions between glutenin subunits occur via intermolecular \( \beta \)-sheets (Popineau et al. 1994). In this study, FTIR analysis was performed on the harvest samples to determine if glyphosate caused significant differences in the secondary structure of proteins after subjecting the samples to mixing as explained in the methods section. Figure 4.1 shows the FTIR profiles for the dough samples corresponding to different treatments.

![FTIR Profiles](image)

Figure 4.1. FTIR profiles of dough samples. The wave length ranges corresponding to different secondary structures is as follows, 1620-1641 nm: \( \beta \)-sheet, 1643-1651: random coil, 1652-1658: \( \alpha \)-helix and 1660-1683: \( \beta \)-turn

As depicted in Figure 4.1, the FTIR profiles of the different samples did not show any differences for the different treatments compared to the controls. Statistical analysis of the percent area of \( \beta \)-sheets, \( \alpha \)-helix, \( \beta \)-turn and random coil structures did not show significant differences between the treatments (glyphosate vs. control), application time (soft dough vs. ripe).
or the interaction between these factors. Thus, the results indicate that glyphosate treatment may not have a profound impact of the secondary structure of gluten proteins. Protein secondary structure formations affect the formation of the gluten network and the protein interactions that occur during the different steps of breadmaking, thus these results indicate that breadmaking may not be impacted by the pre-harvest application of glyphosate. This could be because pre-harvest application of glyphosate does not significantly impact the amino acid composition of wheat, which plays a major role in determining secondary structures. Furthermore, as reported by Bonomi et al. (2013) gluten protein secondary and tertiary structure are determined by genetics, amino acid substitutions or a combination of these factors. When glyphosate is used as a pre-harvest desiccant it is usually applied 1-2 weeks prior to harvest, at which point spring wheat is physiologically mature. At this point, the protein deposition process might be in its very late stages, causing the impact of glyphosate on protein primary and secondary structure to be minimal.

The percent area observed for β-turn ranged between 19 and 40%, whereas the range for α-helical structures was between 9 and 16%. The values for β-sheet and random coil structures ranged between 37 and 49% and 9 to 19% respectively. In a previous study on the secondary structure of hard wheat, the following ranges were obtained; β-turn: 8.2-38.8%, α-helix: 3.6-10.1%, β-sheet: 36.6-50.5%, and random coil/ aperiodic: 14.5-23.7% (Cao et al. 2016). Thus, the values obtained in the current study are in range for what has been observed previously for hard wheat cultivars. In the current study and in that of Cao et al. (2016) β-sheet appeared to be the dominant structure followed by β-turns, random coil and α-helix. Similar observations were made by Jazaeri et al. (2015), Bock et al. (2013) and Marti et al. (2016).
4.4.3. FTIR analysis of wheat flour samples

FTIR analysis of flour samples could be used to determine differences in moisture, lipid, protein and carbohydrate characteristics as previously explained by Marti et al. (2016). Figure 4.2 below shows the FTIR spectra for treated and control samples from T0 (prior to application) until harvest (T3 for ripe and T5 for soft dough).

Figure 4.2. FTIR spectra of whole wheat flour samples. A: soft dough glyphosate; B: soft dough control; C: ripe glyphosate; D: ripe control. T0: prior to application, and T1-T3/T5 indicated the time points corresponding to every three days from application until harvest.

As shown in Figure 4.2, the FTIR spectra for the treated and control samples showed the same pattern. Some differences were observed between T0 and harvest, which could be related to the physiological development taking place during this time frame.
The 3000-3700 cm\(^{-1}\) corresponds to the moisture content of the samples (Marti et al. 2016). The peak at approximately 3400 cm\(^{-1}\) represents the hydrogen bonded water clusters in the flour samples. Differences were observed in this region in the treated and control samples could be attributed to differences in moisture content which ranged from approximately 9% to 13%. The 3000-2800 cm\(^{-1}\) region corresponds to the lipids in flour. Although lipid content was not quantified in this study, the FTIR spectra of the samples show that the lipid content is not drastically different in the treated and the control samples at almost all time points analyzed in this analysis. The peaks in the region of 800-1200 cm\(^{-1}\) represent starch characteristics of flour.

As depicted in Figure 4.2, all samples showed the same shape, however some differences can be observed in the intensity of peaks in this region. As part of this study, starch characteristics such as amylose and amylopectin molecular weight and ratio were investigated, and the results showed that glyphosate did not affect these characteristics. However, the results indicated that glyphosate affected the proportion of A- and B- type starch granules in wheat. Thus, the differences observed in the FTIR spectra could be due the differences in the proportion of A- and B- type starch granules caused by glyphosate. The association between starch granule distribution and FTIR spectra was previously described by Cai et al. (2014). In addition, starch damage that occurs during milling could also contribute to the observed differences as explained by Marti et al. (2016). Changes in protein secondary structure characteristics are represented by peaks in the 1600-1700 cm\(^{-1}\) region. The peaks in this region did not show differences in terms of intensity or shape indicating that protein secondary structure may not be affected by glyphosate as was observed in the previous section. Minor differences could be due to differences in protein content which ranged from 13 – 15% in these samples as analyzed by the Dumas nitrogen analyzer.
4.4.4. SE-HPLC analysis of SDS extractable and unextractable wheat proteins

Changes in the gluten protein profile of whole wheat samples was analyzed by SE-HPLC. The percent area of the different fractions was considered as the proportion of specific proteins, which altogether represents the protein composition. The molecular weight of protein fractions was also analyzed in this experiment. Figure 4.3 shows representative chromatograms for treated and control samples used in this study.

Figure 4.3. SE-HPLC chromatograms for harvest samples. A: SDS extractable proteins; B: SDS unextractable proteins

As shown in Figure 4.3, some differences between the treated and the control samples were observed in terms of peak intensity for SDS extractable and unextractable proteins. Analysis of variance (ANOVA) with mean separation using LSD was used to determine statistically significant differences in the proportion and molecular weight of different protein fractions. The retention times (in min) of the different fractions used in this analysis is as
follows: F1: 3.5-5.2 min, F2: 5.2-5.6 min, F3: 5.6-6.3 min, and F4: 6.3-7.7 min. According to a previous analysis by Larroque et al. (1997), F1-F4 are composed of the following proteins; F1: high molecular weight polymeric proteins, F2: low molecular weight polymeric proteins, F3: gliadin proteins and F4: albumin and globulin.

When determining significant differences between treatments (glyphosate vs. control), application time (soft dough vs. ripe) and the interaction between these factors in the time series analysis (T0 – harvest), significant differences were not observed for any of the extractable (EF1-EF4) or unextractable (UF1-UF4) in the soft dough treated samples. In the ripe treated samples, significant differences were observed for treatment for EF1, UF3 and UF4. For EF1, the control showed higher percent area compared to the control, while it was the opposite for UF3 and UF4. In the harvest samples, significant differences were not observed between the different treatments and control indicating that pre-harvest glyphosate application does not affect wheat gluten protein composition. This is an important observation since specific gluten fractions have been associated with specific functions in dough and baking. For example, many studies have shown the glutenin polymeric proteins have favorable effects on spring wheat dough and baking quality (Malalgoda et al. 2018; Ohm et al. 2009).

The molecular weight of gluten protein fractions was also analyzed in this experiment. In the soft sough treated samples, all fractions except EF2 showed significant differences between treatments, where in general, higher molecular weight values were observed in the control samples (Table 4.2). In the ripe treated samples, significant differences were observed for all gluten protein fractions (EF1-3 and UF1-3). Similar to the observations made for soft dough treated samples, in the ripe treatment too showed higher molecular weight values for controls compared to treated samples. Additionally, for the molecular weight of UF2, significant
differences were also observed for the different time points, and for UF3, significant differences were found between the different time points as well as for the interaction between treatment and time. Thus, the results show that the molecular weight of SDS extractable and unextracted proteins are affected by glyphosate treatment at both application times.

Table 4.2. Treatment averages for the samples collected prior to application until harvest

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein molecular weight (kDa)</th>
<th>Soft dough</th>
<th>Ripe</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glyphosate</td>
<td>Control</td>
<td>Glyphosate</td>
</tr>
<tr>
<td>EF1</td>
<td>4157.6 b</td>
<td>5290.7 a</td>
<td>4455.0 b</td>
</tr>
<tr>
<td>EF2</td>
<td>1225.3 a</td>
<td>1560.1 a</td>
<td>1326.1 b</td>
</tr>
<tr>
<td>EF3</td>
<td>246.4 b</td>
<td>337.5 a</td>
<td>274.8 b</td>
</tr>
<tr>
<td>UF1</td>
<td>900.9 b</td>
<td>1158.3 a</td>
<td>983.8 b</td>
</tr>
<tr>
<td>UF2</td>
<td>443.4 b</td>
<td>695.9 a</td>
<td>527.4 b</td>
</tr>
<tr>
<td>UF3</td>
<td>233.5 b</td>
<td>423.2 a</td>
<td>262.2 b</td>
</tr>
</tbody>
</table>

Values followed by the same letter are not significantly different at $P \leq 0.05$. LSD values compare the glyphosate vs. control in the soft dough and ripe treated samples separately

aEF1-EF3: SDS extractable fractions 1-3, UF1-UF3: SDS unextractable fractions 1-3

The differences in molecular weight observed in the harvest samples are shown in Figure 4.4. Similar to the findings of the time series samples, EF2, EF3 and UF3 showed significant differences for molecular weight in the harvest samples. For all protein fractions, the molecular weight was higher in the control compared to the treated. Nevertheless, the overall results indicate that pre-harvest application of glyphosate affected the molecular weight of wheat gluten protein fractions. In the protein deposition process in wheat, glutenin proteins are deposited late during maturation causing the overall molecular weight to increase over time (Stone and Savin 1999). When glyphosate is applied, it may be interfering with the deposition process, causing molecular weight to decrease. Additionally, in the correlation analysis between gluten protein molecular weight and shikimic acid content, the data showed that there was a significant negative correlation between shikimic acid content and gluten protein molecular weight when glyphosate is applied at soft dough stage, whereas significant correlations were not found
between the two factors at ripe stage. Thus, the effect of reducing molecular weight may be greater when glyphosate is applied at soft dough stage compared to ripe stage.

Many previous studies have highlighted the importance of the molecular weight distribution of wheat proteins in relation to dough functionality (Malalgoda et al. 2018; Ohm et al. 2017). In a review by Southan and MacRitchie (1999) genetic and environmental factors were highlighted as contributors to changing the properties of wheat gluten proteins. According to the review, nine major loci (three Glu-1, three Gli-1/ Glu-3 and thee Gli-2) determine the functional attributes of wheat proteins, and allelic variation can be found at each of these loci. Additionally, environmental factors such as availability of nitrogen, sulfur and temperature conditions during the physiological development of wheat can affect the chemistry of gluten proteins. There has not been any studies investigating the application of pre-harvest desiccant on gluten protein molecular weight, thus the current study serves as one of the first in this area.
4.4.5. Shikimic acid accumulation

Glyphosate inhibits a key enzyme in the shikimic acid pathway in plants as its mode of action (Williams et al. 2000). Shikimic acid accumulates as a result of glyphosate application because the negative feedback controlling the level of shikimic acid is interrupted by the inhibition of the enolpyruvylshikimate-3-phosphate enzyme. That is, glyphosate causes an unregulated flow of carbon to intermediates, such as shikimic acid that are found prior to the inhibition point. Table 4.3 shows the levels of shikimic acid detected in samples collected prior to application until harvest for soft dough and ripe treated samples separately.
Table 4.3. Accumulation of shikimic acid over time<sup>a</sup>

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time point</th>
<th>Shikimic acid level (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Soft dough</td>
</tr>
<tr>
<td>Glyphosate</td>
<td>T0</td>
<td>13.2 a</td>
</tr>
<tr>
<td></td>
<td>T1</td>
<td>56.0 a</td>
</tr>
<tr>
<td></td>
<td>T2</td>
<td>81.5 a</td>
</tr>
<tr>
<td></td>
<td>T3</td>
<td>92.8 a</td>
</tr>
<tr>
<td></td>
<td>T4</td>
<td>104.7 a</td>
</tr>
<tr>
<td></td>
<td>T5</td>
<td>131.3 a</td>
</tr>
<tr>
<td>Control/ water</td>
<td>T0</td>
<td>13.0 a</td>
</tr>
<tr>
<td></td>
<td>T1</td>
<td>14.4 b</td>
</tr>
<tr>
<td></td>
<td>T2</td>
<td>14.1 b</td>
</tr>
<tr>
<td></td>
<td>T3</td>
<td>11.6 b</td>
</tr>
<tr>
<td></td>
<td>T4</td>
<td>14.3 b</td>
</tr>
<tr>
<td></td>
<td>T5</td>
<td>12.6 b</td>
</tr>
<tr>
<td>Overall treatment</td>
<td>Glyphosate</td>
<td>79.9 a</td>
</tr>
<tr>
<td></td>
<td>Control/ water</td>
<td>13.3 b</td>
</tr>
</tbody>
</table>

<sup>a</sup>LSD values compared glyphosate vs. control (glyphosate T0 vs. control T0). Values followed by the same letter are not significantly different <i>P</i> ≤ 0.05

As shown in Table 4.3, for the samples treated at soft dough, except at T0, glyphosate treated samples showed significantly higher amount of shikimic acid compared to the control. However, samples treated at ripe, significant differences were not observed between the treatment and control. Thus, shikimic acid accumulation is highly significant when glyphosate is applied at soft dough stage compared to application at ripe stage. In the analysis of the harvest samples, significant differences were observed in the level of shikimic acid, where the amount was significantly higher in the samples treated at soft dough compared to the ripe treated samples and the control samples. Previous studies have also noted shikimic acid accumulation as a result of pre-harvest application of glyphosate in spring wheat (Bresnahan et al. 2003). In other plants species too similar observations have been made (Becerra-Moreno et al. 2012; Buehring et al. 2007; Mueller et al. 2003). Anderson et al. (2001) determined that shikimic acid accumulation is directly proportional to the amount of glyphosate application, further highlighting the impact of
the two factors. Determining the level of shikimic acid accumulation has been suggested as a method to test for glyphosate efficacy (Harring et al. 1998). In this study, the accumulation of shikimic acid when glyphosate is applied at soft dough stage can be represented as shown in Figure 4.5. A somewhat similar trend was observed by Bresnahan et al. (2003), where shikimic acid accumulation peaked 3-7 days after treatment and declined thereafter until harvest.

![Figure 4.5. Accumulation of shikimic acid when applied at soft dough stage](image)

\[ y = 18.87 + 38.6 \times \ln(\text{Time}) \]
\[ R^2 = 0.966 \]

4.5. Conclusions

Glyphosate is the most widely used herbicide in the world, and it is sometimes used as a pre-harvest desiccant or harvest-aid during wheat cultivation. In this case, glyphosate is applied right before harvest to accelerate the process of crop maturation and desiccation. In this experiment, the effect of pre-harvest application of glyphosate on spring wheat gluten protein properties was investigated. Glyphosate was applied at soft dough and ripe stages of physiological maturity, and samples were collected prior to application and every three days until harvest. The wheat samples were then milled using a laboratory scale mill to obtain whole wheat flour, after which the amino acid composition, protein secondary structure, gluten protein...
composition, gluten protein molecular weight and shikimic acid level was determined using techniques such as FTIR, SE-HPLC and RP-HPLC. The results of the protein primary and secondary structure analysis showed that glyphosate application does not have a significant impact on changing the amino acid composition or the secondary protein structure of wheat gluten proteins. The FTIR analysis of flour samples indicated that carbohydrate related characteristics may be impacted by glyphosate, but did not show differences for protein or lipid related characteristics. The SE-HPLC analysis of SDS extractable and unextractable proteins showed that glyphosate does not affect gluten protein composition, however, significant differences were observed for the molecular weight of mainly SDS extractable and unextractable proteins. Furthermore, there were indications that the effects may be more pronounced when application is done at soft dough stage. Shikimic acid accumulation as a response to glyphosate treatment was found to be highly significant when applied at soft dough stage. Significant differences were not found when applied at ripe stage. The results showed that the rate of accumulation increased rapidly in the first few days and then reached a plateau like stage later on. Overall, the results of this study indicate that glyphosate application impacts gluten protein molecular weight and shikimic acid accumulation when applied at soft dough stage, in a significant manner, although other protein primary and secondary did not show significant differences. However, when glyphosate was applied at the recommended stage significant effects were not found. The observed differences in protein molecular weight could be due to glyphosate’s effect of wheat protein biosynthesis and the effect of deregulation of the shikimic acid pathway and subsequent effects on the carbon flow of affected plants.
4.6. References


CHAPTER 5. GLYPHOSATE TIMING AND EFFECTS ON THE FINE CHEMISTRY
OF WHEAT STARCH

5.1. Abstract

Glyphosate is the most widely used herbicide in the world, and it is used as a pre-harvest desiccant or harvest aid during wheat cultivation. Glyphosate is recommended to be applied at the ripe stage of physiological maturation when it is used as a pre-harvest desiccant during wheat cultivation. In this context, the aim of this study was to determine if glyphosate application at different developmental stages (early and late) has an effect on the physicochemical properties of wheat starch. For this purpose, a greenhouse study was conducted where glyphosate was sprayed at ripe stage and soft dough stage to a commonly grown wheat cultivar in North Dakota. Samples were collected before application and every three days after application until harvest. The controls used in the study were sprayed with water instead of glyphosate at the same developmental stages. The obtained samples were milled then analyzed for differences in starch granule distribution, amylose and amylopectin ratio, amylose and amylopectin molecular weight, starch granule morphology, amylopectin chain length and starch thermal properties. The results of the study indicate that glyphosate does not impact the spatial distribution and morphology of starch granules. The percentage of amylose and amylopectin, amylose and amylopectin molecular weight did not show significant differences between treatments. However, the results showed that glyphosate affected the thermal characteristics of wheat starch when applied at soft dough stage and that it decreased the average amylopectin chain length. Differences in the proportion of short, medium and long chain amylopectin was observed in both treatments. Overall, this study shows that glyphosate has some effects on wheat starch properties, especially
when applied at soft dough stage, although it is unclear how these changes affect overall functionality of wheat starch in food systems.

5.2. Introductions

Starch accounts for approximately 54-72% of the dry weight of wheat kernels (Stone and Morell 2009). It has been found that starch content in wheat is positively associated with yield, but negatively associated with protein content (Hucl and Chibbar 1996). In wheat, starch is synthesized in specialized organelles termed amyloplasts. Each wheat amyloplast contains one starch granule (Bechtel and Wilson 2000; Bechtel et al. 1990; Parker 1985). In mature wheat starch, two types of granules, A-type (large and lenticular) and B-type (small and spherical), can be found, where the former is produced following a week after anthesis and the latter is produced 2-3 weeks after anthesis (Bechtel et al. 1990). The diameter of A-type of granules is longer than 16 µm, whereas the B-type granules are between 5 – 16 µm. At harvest, wheat starch contains approximately 30% B-type granules by weight, and by number these granules account for 90-97% (Maningat et al. 2009). Wheat starch granule size distribution is bimodal, with other cereals such as barley, rye and triticale having similar distributions. Moreover, granule size distribution is largely controlled by genetic factors; however, environmental factors such as drought and agricultural practices have shown to decrease the proportion of A-type starch granules (Zhang et al. 2017). In contrast, growing conditions with comparatively higher number of grain filling days with temperatures above 30°C resulted in wheat grains having higher proportions of A-type starch granules (Liu et al. 2011; Panozzo and Eagles 1998).

Wheat starch is composed of 25% amylose and 75% amyllopectin (Maningat et al. 2009). Amylose is essentially a linear polymer with 0.2-0.8% of branches, and the degree of polymerization (DP) of amylose is between 1,000 and 5,000. Amylopectin is a large branched
molecule with 4-6% linkages, with DP values over 10,000. Previous work on amylopectin has shown that these polymers account for 70-80% of most starches, and that the amylopectin fraction is important in determining characteristics such as crystallinity, birefringence, swelling and gelatinization and the growth rings formed in starch granules. Additionally, amylopectin has been found to be esterified with low levels of phosphate groups. The molecular weight of wheat amylopectin is estimated to be between $2.6 \times 10^8$ Da and $7.0 \times 10^8$ Da according to light scattering analysis of starch molecular weight (Franco Célia et al. 2002). Amylopectin chains are categorized as A, B or C type chains (Maningat et al. 2009). A-type chains are linear and do not contain branch points, B-type chains carry A- and/or C-type chains, and C-type chains carry the single reducing end found in amylopectin. Previous work on amylopectin chain length distribution has determined that wheat starch contains 20%, 43%, 16% and 18% respectively of A, B$_1$, B$_2$ and B$_n$ type chains (percentages are based on weight percentages of chains) (Franco Célia et al. 2002; Yoo and Jane 2002b).

As mentioned earlier, glyphosate, which is an herbicide used as a pre-harvest desiccant during wheat cultivation. It is applied prior to harvest to accelerate the process of crop maturation and reduce the moisture content of the grains which allows for timely harvest. In recent years, the use of glyphosate has increased rapidly due to factors such as the introduction of glyphosate resistant crops (soybean and corn) and evolving agricultural practices like pre-harvest use of herbicides for desiccation (Benbrook 2016). Glyphosate inhibits a key enzyme in the shikimic acid pathway disrupting the biosynthesis of aromatic amino acids, thereby causes the death of the affected crops (Williams et al. 2000). That is, glyphosate inactivates the enolpyruvylshikimate phosphate synthase enzyme which catalyzes the biochemical reaction that converts shikimate/shikimic acid to 3-enolpyruvylshikimate-5-phosphate. The latter is the
precursor to chorismite, which is later converted into aromatic amino acids. Some studies have shown that glyphosate disrupts the carbon flow of affected plants because it causes an accumulation of shikimic acid, which is a precursor of aromatic amino acids as explained earlier (Zobiole et al. 2011).

Glyphosate’s effects on plant physiology have been investigated in the last few decades and many studies have shown the glyphosate causes losses in yield and reduces seedling emergence rate (Yenish and Young 2000). Some researchers have also found that it also affects the photosynthetic apparatus of affected plants by way of preventing/ decreasing the synthesis of secondary metabolites such as quinones (Fedtke and Duke 2005; Zobiole et al. 2012). Furthermore, glyphosate’s ability to chelate metal ions has also been implicated its negative effects on photosynthesis (Cakmak et al. 2009). For instance, glyphosate could bind metal ions such as magnesium which is essential for the biochemical reactions involved in photosynthesis. Servaites et al. (1987) who studied the effect of glyphosate on carbon fixing in sugar beet found that glyphosate can reduce the level of ribulose-1, 5-biphosphate (RuBP) and 3-phosphoglyceric acid, and the enzyme ribulose-1,5-biphosphate carboxylase which leads to reduced carbon fixing during the process of photosynthesis. This study further highlighted that glyphosate could reduce the rate of starch synthesis in sugar beet although it did not have a profound effect on the biosynthesis and translocation of sucrose.

As previous studies mentioned above show, glyphosate can affect the starch synthesis of affected plants by interfering with the carbon flow due to the accumulation of shikimic acid, availability of metal ions, reducing the levels of substrate molecules used in photosynthesis and by reducing enzyme activity. In wheat, since glyphosate is applied during the process of physiological maturation, it could interference with the deposition and chemistry of wheat starch.
In this context, the goal of this study was to examine how glyphosate application time affects the fine chemistry of wheat starch in order to predict what such quality changes may occur in final wheat-based products.

5.3. Materials and methods

5.3.1. Design of greenhouse experiment

Glenn, which is one of the most widely grown wheat cultivars adapted to the conditions in the Northern Plains was used in this study. A germination test was performed under laboratory conditions to determine the germination rate of the seeds, which was found to be over 90%. A randomized complete block design was used for the greenhouse experiment with four different treatments, namely, glyphosate sprayed at soft dough stage, glyphosate sprayed at ripe stage of maturity, and the control for each of the two treatments, where water was sprayed instead of glyphosate at soft dough and ripe stages. A single seed was placed in each planting cone containing soil (PRO-MIX® LP15, Premier Tech Horticulture, Quakertown, PA). Multicote™ (Haifa Group, Altamonte Springs, FL) slow release fertilizer was applied at a rate of 1/8th of a teaspoon two weeks after planting. The wheat plants were watered frequently, and the growth of the plants was monitored. When soft dough stage was reached (contents of the kernel is no longer milky, it has solid like texture), a spray booth (Generation III Research Sprayer, Devries Manufacturing, Hollandale, MN) was used to spray glyphosate at a rate of 1.1 kg of active ingredient/ha. As mentioned earlier, for the control soft dough treatment, water was sprayed in place of glyphosate. The sprayed plants were taken back to the greenhouse room approximately 4 hours after spraying. The same was performed when ripe stage (kernels are harder than at soft dough stage and indented with the finger nail a slight dent remains) was reached. For the two glyphosate treatments and the water controls, wheat heads were collected prior to spraying, one
day after spraying, and every three days until harvest (T0-T5 for soft dough, T0-T3 for ripe). The heads were dried at 30°C for 2 days in a forced air oven (Shel Lab, Sheldon Manufacturing, Inc. Cornelius, OR) then threshed using a wheat head thresher (Precision Machine Co. Inc., Lincoln, NE). The wheat kernels were then milled using a ball mill (Retsch Mixer Mill MM400, Retsch, Haan, Germany) at a frequency 30/s of for 2 min. The whole wheat samples were stored at -20°C until further analyses.

5.3.2. Spatial distribution of starch granules

Kernels were fixed in 10% neutral buffered formalin for 24 hours, then bisected and fixed further for another 24 hours. The kernels were processed for 2 hours in the following order, 70%, 80%, 95% 100%, 100% ethanol, xylene, xylene, paraffin, paraffin and paraffin. The kernels were then embedded in paraffin on a Sakura Tissue Tek TEC5 (Sakura Finetek USA, Inc, Torrance, CA). Slides were then cut put on a Leica RM2255 microtome (Leica Biosystems Inc., Buffalo Grove, IL), at 5 µm thickness, and baked for 30 min at 60°C. Afterwards, the slides were deparaffinzed, and stained with Lugol’s solution for 5 min, and washed with water to remove any excess staining. The slides were then dehydrated, cleared and then mounted. The slides were imaged using a MoticEasyScan digital slide scanner (Motic, British Columbia, Canada) at x40 magnification, and the Image Pro Premier 9.3 software was used to determine distribution of starch granules.

5.3.3. Analysis of amylose and amylopectin percentages and molecular weight

For the extraction of starch 30 – 40 mg flour was used. The samples were first defatted by adding 2.5 mL of methanol and heating at 100°C for 30 min. The samples were then centrifuged at 2,000 rpm for 5 min (Allegra X-12, Beckman Coulter, Indianapolis, IN, U.S.A). Afterwards, the supernatant was discarded and the pellet was dried at 55°C. To extract starch, 2 mL of
potassium hydroxide/urea (4.5 mL of 1M KOH with 0.5 mL of 6M urea) was added and heated for 15 min at 100℃.

A method previously described by Grant et al. (2002) was used to prepare samples for SE-HPLC analysis. A volume of 4.5 mL of 1M KOH and 0.5 mL of urea was added to the samples and heated for 90 min at 100℃. After the samples cooled to room temperature, a 1 mL aliquot of sample was neutralized with 1M HCl and subsequently filtered through a 0.45 µm nylon membrane (VWR International). An Agilent 1200 series HPLC system (Agilent Technologies, Santa Clara, CA, U.S.A.) which was connected to an auto sample, refractive index (RI) detector, and a Wyatt Dawn Helios-II multi-angle light scattering (MALS) detector was used in the analysis. A Waters Ultrahydrogel guard column (6 µm, 6 mm x 40 mm), and Ultrahydrogel 1000 (12 µm, 7.8 mm x 300 mm) and a linear size exclusion column (10 µm, 7.8 mm x 300 mm) (Waters, Milford, MA, U.S.A.) were used for the separation of amylose and amyllopectin. The HPLC analysis was performed at 40℃ at a flor rate of 0.3 mL/min and the injection volume was 60 µL. The mobile phase used in this experiment was water.

To determine the amylose and amyllopectin percentages the ChemStation software (HP ChemStation for LC Rev. A.04.01) was used. To determine the molecular weight of amylose and amyllopectin the Astra 6.0.5 software (Wyatt Technology Corporation, Santa Barbara, CA, U.S.A.) was used. As per the findings of You et al. (1999) a dn/dc value of 0.146 was used and the data was normalized using pullulan standards followed by baseline corrections and peak alignments. The Debye model with a fit degree of 2 and a first-order polynomial fit were applied using the calculations.
5.3.4. Extraction of starch from whole wheat samples

To extract starch from whole wheat flour a sieving technique was used. Starch was extracted from samples collected prior to harvest (T0), samples at the mid-point between application and harvest (Tm) and samples collected at harvest (Te). A volume of 50 mL of deionized water was added to 10 g of flour and blended for 3 min using an electric hand blender. Four sieves with decreasing sieve sizes (Tyler sieve numbers 60, 120, 200, 325) were stacked with the largest pore size on top and the smallest at the bottom. This method is similar to method described by Dik et al. (2002) who extracted wheat starch from dough. The stack of sieves was placed on a clean tray to collect the starch slurry. The blended mixture was poured on the stack of sieves and a spatula was used to wash the flour particles while spraying deionized water. After the starch passed through the first sieve, the same washing procedure was performed and for each sieve until the last tray was reached. The starch slurry was transferred (approximately 200-250 mL) into a centrifuge bottle and centrifuged at 3,000 x g for 5 min (Allegra X-12, Beckman Coulter, Indianapolis, IN, U.S.A). The supernatant was discarded, and the pellet was transferred into two 50 mL round-bottom centrifuge tubes and filled with deionized water about half way through, then centrifuged at 15,000 x g for 10 min (Allegra 64R centrifuge, Nackman Coulter). Afterwards, the supernatant was discarded and the two starch pellets (from the two tubes) was placed on 1 No. 325 Tyler sieve (kept on a beaker) and washed again with deionized water without destructing the pellets. The starch slurry collected in the beaker was transferred into clean round-bottom centrifuge tubes and centrifuged again at 15,000 x g for 10 min (Allegra 64R centrifuge, Nackman Coulter). The supernatant was discarded. A volume of 20 mL of water was added to the pellet, re-suspended and centrifuged at the same conditions. The supernatant was discarded and the water washing repeated two more times. Afterwards the pellet was washed
with 95% ethanol under same conditions, and finally acetone was used to wash the pellet. The starch pellet was then transferred on to a glass watch glass and dried overnight under a fume hood. The following day, the starch pellet was ground using a motor was pestle.

5.3.5. Scanning electron microscopy analysis of extracted starch

Samples were applied to adhesive carbon tabs on cylindrical aluminum mounts and the excess blown off with a stream of nitrogen gas. Samples were coated with a conductive layer of gold using a Cressington 108auto sputter coater (Ted Pella Inc., Redding CA, USA). Images were obtained using a JEOL JSM-6490LV scanning electron microscope (JEOL USA, Peabody MA, USA) at an accelerating voltage of 15 kV.

5.3.6. Analysis of thermal properties using differential scanning calorimetry (DSC)

A Perkin-Elmer Differential Scanning Calorimeter DSC-7 was used to determine the thermal characteristics of the extracted wheat starch following a method previously described by Kim et al. (1997) with modifications. Prior to the day of analysis, 3.5 mg of each sample was weighed into aluminum pants and 8 µL of deionized water was added. The pans were then hermetically sealed and kept overnight. To determine thermal characteristics such as enthalpy of gelatinization, onset temperature, peak temperature and end temperature, the samples were heated at 10°C/min from 10 to 100°C. During the analysis an empty reference pan was placed next to the pan containing the sample. The data processing software on the DSC was used to obtain the thermal characteristics mentioned earlier.

5.3.7. Separation of amylose and amylopectin using gel permeation chromatography (GPC)

Extracted starch (30 mg) was dissolved in 3 mL of 2M NaOH was kept stirring at 70°C for 2 hours, and subsequently filtered through a 1.5 µm nylon syringe filter (Patindol et al. 2007).
The sample was then loaded on to a GPC column (1.6 x 100 cm, Pharmacia Inc, Piscataway, NJ) which was packed with Sephadex CL-2B gel (Sigma-Aldrich, St. Louise, MO). The mobile phase was 10 mM NaOH, and the flow rate was 0.4 mL/min. Fractions were collected every 9 min for 18 hours on a fraction collector (Spectra/Chrom® CF-1 Fraction Collector, Spectrum Chromatography, Houston, TX). Approximately 120 tubes were collected and every third tube was used to perform the blue value to identify the amylose and amylopectin fractions and the phenol-sulfuric assay was done to determine total carbohydrates.

To perform the blue value assay 100 µL of I₂/KI solution (0.2 g of I₂ and 2 g of KI in 100 mL of 0.1M phosphate buffer, pH 5) was added to 100 µL sample. The absorbance was then read at 580 nm. To perform the phenol-sulfuric assay, 100 µL of 5% phenol was added to 100 µL of sample and mixed. Then 0.5 mL of concentrated sulfuric acid was added, vortexed and boiled for 10 min. The absorbance of the sample was read at 492 nm. Following the assays, the fractions containing amylopectin were pooled and dialyzed against distilled water at 4°C for 3 days. Afterwards the dialyzed amylopectin fraction was freeze dried and used to determine chain length distribution.

5.3.8. **Determination of amylopectin chain length**

To prepare samples for chain length analysis a method previously described by Patindol et al. (2007) was used. The freeze-dried amylopectin fraction (2 – 4 mg) was boiled (with stirring) with 3.2 mL of deionized water for 1 hour, and upon cooling 0.4 mL of 0.1 M acetate buffer, pH 3.5 and 40 µL of isoamylase (500 U/mL, Megazyme) were added. The samples were kept stirring at 40°C for 48 hours. To stop the enzyme activity, the samples were then boiled again for 20 min. To remove any colloidal material, the samples were then centrifuged at 5,000 rpm, and 12 µL of the supernatant was injected for analysis. The chain length distribution was
analyzed using a Dionex ICS5000+ high performance anion exchange chromatography equipped with a pulsed amperometric detector (HPAEC-PAD). A CarboPac PA-100 column was used for separation. Two elution solvents 100 mM NaOH (Solvent A) and 400 mM sodium acetate in 100 mM NaOH (Solvent B) were used during the experiment. The linear gradient of the solvents was as follows, 0 – 30 min: 0 - 62.5% B, 31-45 min: 62.5 – 100% B. An electrochemical detector with gold electrode in pulsed amperometric mode was used to detect the signal. The gold carbo quad waveform (0.4s 0.1V, 0.02s -2.0V, 0.01s 0.6V, 0.07S -0.1V) was used for detection, and the amylopectin chain length distribution was calculated based on the total area percentage.

5.3.9. Statistical analysis

The SAS software (Version 9.3, SAS Institute, Cary, NC) was used to perform Analysis of Variance (ANOVA) and mean separation (Fisher’s protected LSD).

5.4. Results and discussions

The results of the starch tests were analyzed in two different ways. First, the two treatments (soft dough and ripe) were analyzed separately to determine the effect of treatment (glyphosate vs. water) and time (if the level is different between the different time points). Next the harvest data was analyzed to determine significant differences between application time (soft dough vs. ripe) and treatment (glyphosate vs. control).

5.4.1. Spatial distribution of starch granules

To determine if glyphosate application time affects starch granule distribution, staining experiments were performed in this study. Differences were not observed in the spatial distribution of starch granules prior to application, and at the subsequent time points until harvest indicating that glyphosate application does not affect the spatial distribution of wheat starch granules. However, glyphosate may cause changes in the fine chemistry of starch at molecular
Figure 5.1 shows the distribution of granules in the harvest samples of the different treatments.

Figure 5.1. Spatial distribution of wheat starch granules in harvest samples. A: Soft dough glyphosate treatment, B: Ripe glyphosate treatment, C: Control soft dough treatment, D: Control ripe treatment
5.4.2. Starch granule analysis using scanning electron microscopy

The starch extracted from whole wheat samples was analyzed using scanning electron microscopy to determine changes in starch granule morphology. Figure 5.2 shows the observations made during this experiment.

Figure 5.2. Scanning electron microscopy images of extracted starch. 1: glyphosate treatment at soft dough stage, 2: glyphosate treatment at ripe stage, 3: control soft dough treatment and 4: control ripe treatment; A: $T_o$, B: $T_m$, and C: $T_e$
As shown in Figure 5.2, two types of starch granules, A-type and B-type were observed in all treatments at the three different time points. The granules appeared to be intact and did not show evidence of starch damage in any of the treatments. The morphological features of starch granules did not show differences between treatments or time points indicating that glyphosate treatment does not impact starch granule morphology.

In previous work regarding factors affecting starch granule morphology, Singh et al. (2007) showed that chemical modifications such as, hydroxypopylation can cause potato starch granules to fold and form doughnut like structures. Additionally, deep grooves were also observed as a result of modification. Hoover and Vasanthan (1994) studied the effect of heat-moisture treatment on the granule morphology of cereal starches and found that wheat starch characteristics do not change as a result of such treatments, whereas oat granules become less compact. Similarly, annealing also leads to similar observations in wheat and oat starches (Hoover and Vasanthan 1993). In a similar study conducted by Stebbins (2018), glyphosate application did not cause changes in oat granule morphology, which is in agreement with the observations made in the present study. Thus, the results show that glyphosate treatment does not affect starch at granular level, although wheat starch might be affected by glyphosate at molecular level.

5.4.3. Amylose-to-amylopectin ratio and molecular weight

In this study, SE-HPLC analysis of extracted starch was used to determine changes in the percentage of amylopectin and amylose, and the molecular weight of these polymers. Table 5.1 shows the percentage data for starch polymers for the different time points and the overall treatments.
Table 5.1. Amylopectin and amylose percentage<sup>a</sup>

<table>
<thead>
<tr>
<th>Treatment</th>
<th></th>
<th>% of amylopectin</th>
<th></th>
<th>% of amylose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Soft dough</td>
<td>Ripe</td>
<td>Soft dough</td>
</tr>
<tr>
<td>Glyphosate</td>
<td>T0</td>
<td>75.1 a</td>
<td>24.9 a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>T1</td>
<td>75.0 a</td>
<td>25.0 a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>T2</td>
<td>75.7 a</td>
<td>75.2 a</td>
<td>24.3 b</td>
</tr>
<tr>
<td></td>
<td>T3</td>
<td>75.3 a</td>
<td>74.9 a</td>
<td>24.7 a</td>
</tr>
<tr>
<td></td>
<td>T4</td>
<td>75.6 a</td>
<td>75.4 a</td>
<td>24.4 a</td>
</tr>
<tr>
<td></td>
<td>T5</td>
<td>75.4 a</td>
<td>75.2 a</td>
<td>24.6 a</td>
</tr>
<tr>
<td>Control/ water</td>
<td>T0</td>
<td>75.2 a</td>
<td></td>
<td>24.8 a</td>
</tr>
<tr>
<td></td>
<td>T1</td>
<td>75.0 a</td>
<td></td>
<td>25.0 a</td>
</tr>
<tr>
<td></td>
<td>T2</td>
<td>74.8 b</td>
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<td>T5</td>
<td>75.3 a</td>
<td>75.3 a</td>
<td>24.7 a</td>
</tr>
</tbody>
</table>

<sup>a</sup>For the time series data, the LSD values show if there is a significant difference at each time point for the treatment vs. control (T0 glyphosate vs. T0 control).

In the time series analysis, as shown in Table 5.1, significant differences were found for the percentage of amylose and amylopectin for the soft dough application time between the glyphosate treated sample and the control, where the glyphosate treated samples showed higher percentage of amylopectin compared to the control, and vice-versa for amylose. As for differences for individual time points, only T2 showed significant differences for the percentage of amylose and amylopectin. Although these values are statistically significant, it is reasonable to assume that they are similar for practical purposes. For the ripe treatment, significant differences were not observed between the control and treatment with reference to the individual time points or the overall averages. Therefore, these results suggest early application of glyphosate may cause changes to the proportion of amylose and amylopectin in wheat. Additionally, the percentage values observed in this experiment fall within range for previously determined
values, which is 25% for amylose and 75% for amylopectin (Ball et al. 1998; Medcalf and Gilles 1965). The molecular weight of amylopectin and amylose was also determined in this study, and presented in Table 5.2.

Table 5.2. Amylopectin and amylose molecular weight

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time point</th>
<th>Amylopectin (Da)</th>
<th>Amylose (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Soft dough</td>
<td>Ripe</td>
</tr>
<tr>
<td>Glyphosate</td>
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<td>1.16 x 10^7 a</td>
<td>2.44 x 10^6 a</td>
</tr>
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<td>1.16 x 10^7 a</td>
</tr>
<tr>
<td>Overall treatment</td>
<td>Glyphosate</td>
<td>1.10 x 10^7 a</td>
<td>1.13 x 10^7 a</td>
</tr>
<tr>
<td></td>
<td>Control/ water</td>
<td>1.24 x 10^7 a</td>
<td>1.16 x 10^7 a</td>
</tr>
</tbody>
</table>

*aFor the time series data, the LSD values show if there is a significant difference at each time point for the treatment vs. control (T0 glyphosate vs. T0 control).

As shown in Table 5.2, the molecular weight of amylopectin and amylose was not affected by glyphosate treatment, and did not show significant differences between the treatment and the control at the different time points as well as the overall average. The values obtained in this study for the molecular weight of amylopectin and amylose appears to fall within the ranges described in previous studies. For example, the molecular weight of amylopectin has been found to be between 10 and 310 million Da, while the molecular weight of amylose has been determined as 0.1 - 0.2 million Da (Ball et al. 1998; Simsek et al. 2014; Yoo and Jane 2002a).

Additionally, Gidley et al. (2010) determined that the molecular weight of starch polymers is very broad, which could range between 10^5 to 10^6 Da for amylose and 10^7 – 10^9 Da for...
amylopectin. In this study, the molecular weight of amylopectin was between 10 and 15 million Da, while for amylose it was between 2.2 and 3.3 million Da. The percentage of amylose and amylopectin and the molecular weight changes in the harvest samples of the study are depicted in Figure 5.3.

Figure 5.3. Amylopectin and amylose molecular weight in harvest samples

As shown in Figure 5.3, glyphosate did not cause significant changes in amylopectin and amylose molecular weight, which is in agreements with the findings of the previous field study presented in Chapter 3 of this dissertation. Several environmental factors have been investigated as potential causes for changes in wheat starch chemistry. For example, Zhang et al. (2017) studied the effect of water availability and found that amylose biosynthesis is affected to a greater extent in comparison to the biosynthesis of amylopectin in environments with low water availability. However, this study did not investigate the effects on the percentage and the molecular weight of the different starch polymers. Furthermore, in a review by Thitisaksakul et al. (2012) several environmental factors such as drought, heat, salinity, nitrogen deficiency and high levels of CO₂ are discussed as factors affecting the biosynthesis and chemical properties of
cereal starches. Previous studies on different herbicides and starch have shown that starch accumulation can be reduced in proportion to the rate of application (Kumar 2012). Moreover, it has been found that glyphosate can decrease chlorophyll synthesis which could result in reduced levels of starch accumulation (Fedtke and Duke 2005) and that the herbicide isoproturon can cause similar effects (Yin et al. 2008). In terms of the biochemical process of starch synthesis, four key enzymes are required for the process, namely ADP-glucose pyrophosphorylase, starch synthase, starch branching enzymes, and starch debranching enzymes. In this context, glyphosate’s ability to chelate metal ions could interfere with the activation of such enzymes, which require metal cofactors leading to decreases in the rate of starch synthesis (Cakmak et al. 2009; Gomes et al. 2014). According to the observations made in the current study, glyphosate application may not affect amylopectin and amylose molecular weight because starch polymers are very large and changes in the mechanism of starch biosynthesis may not cause significant changes to the overall molecular weight. However, there have not been any previous studies in this area, therefore interactions between glyphosate (and other pre-harvest desiccants as well as herbicides) and starch synthesizing enzymes in cereals need to be investigated furthermore.

5.4.4. Starch thermal properties analyzed using DSC

To analyze the thermal properties of wheat starch treated with glyphosate at the soft dough stage and ripe stages, starch was extracted from whole wheat flour using a sieves of different sizes as described in the methods. Samples obtained prior to application, the mid-point between application and harvest, and harvest were analyzed for differences in thermal characteristics. These results are shown in Table 5.3.
Table 5.3. Thermal properties of glyphosate treated wheat

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Timepoint</th>
<th>Onset temperature (℃)</th>
<th>Peak temperature (℃)</th>
<th>Enthalpy (J/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Soft dough</td>
<td>Ripe</td>
<td>Soft dough</td>
</tr>
<tr>
<td>Glyphosate</td>
<td>T0</td>
<td>59.16 a</td>
<td>58.60 a</td>
<td>63.91 a</td>
</tr>
<tr>
<td></td>
<td>Tm</td>
<td>58.66 a</td>
<td>58.39 a</td>
<td>63.90 a</td>
</tr>
<tr>
<td></td>
<td>Te</td>
<td>58.07 a</td>
<td>58.55 a</td>
<td>63.40 a</td>
</tr>
<tr>
<td>Water/ control</td>
<td>T0</td>
<td>58.35 a</td>
<td>57.97 a</td>
<td>63.44 a</td>
</tr>
<tr>
<td></td>
<td>Tm</td>
<td>57.84 a</td>
<td>58.44 a</td>
<td>63.26 a</td>
</tr>
<tr>
<td></td>
<td>Te</td>
<td>58.46 a</td>
<td>58.30 a</td>
<td>63.57 a</td>
</tr>
<tr>
<td>Overall treatment</td>
<td>Glyphosate</td>
<td>58.63 a</td>
<td>58.51 a</td>
<td>63.73 a</td>
</tr>
<tr>
<td></td>
<td>Water</td>
<td>58.21 a</td>
<td>58.24 a</td>
<td>63.42 a</td>
</tr>
</tbody>
</table>

aFor the time series data, the LSD values show if there is a significant difference at each time point for the treatment vs. control (T0 glyphosate vs. T0 control).

As Yu and Christie (2001) explained, differential scanning calorimetry has been used to measure the thermal characteristics of starches, which include gelatinization properties, glass transition temperature, and crystallization. However, the authors also highlight that the results reported for these properties are not consistent among different studies due to factors such as, the complex nature of the thermal behavior of starches as well as differing measurements conditions. Moreover, the physicochemical changes take place during the heating of starch are very complex, which include chemical changes such as gelatinization, melting, glass transition, crystallization, changes to the crystal structure, volume expansion, degradation and water associated changes such as the motion/migration of water. According to Yu and Christine (2001), these changes are highly associated with the moisture content of starch, which is unstable during the heating process. To add to the complexity, the thermal conductivity of starch is very poor in the context of starch granules, as the bulk density of granules is low. As shown in Table 5.4, glyphosate application at soft dough stage or ripe stage did not cause significant differences in the thermal properties of extracted wheat starch, compared to application of water/control at
the same physiological stages of maturity. This could be because factors affecting the thermal behavior of starches is determined prior to the application stages (soft dough and ripe), thus glyphosate application does not have a profound effect. Additionally, minor differences in the chemical nature of starch caused by glyphosate may not impact of the overall thermal characteristics of wheat starch due to the complex nature of the thermal behavior of starch as explained by Yu and Christie (2001). In a review by Lund (1984), the gelatinization characteristics of wheat starch analyzed by DSC was reported as 52°C, 59°C and 2.4 (cal/g) for onset temperature, peak temperature and enthalpy respectively. In another study by Fujita et al. (1992) the peak temperature range was reported as 59 - 64°C and the enthalpy was determined as 7.4 – 14.1 J/g. Thus, the results of the current study are within the ranges found in previous work in the same area. However, differences between the current study and the results of Lund (1984) could be due to multiple reasons such as, source of wheat starch/ wheat cultivar, method of starch extraction and preparation and DSC conditions. Additionally, as reported by Lund (1984) temperature conditions, moisture content of the sample, presence of surfactants, lipids and other solutes as well as process conditions can affect the gelatinization characteristics. Changes to wheat starch gelatinization properties have not been studied in the context of different herbicides or pre-harvest desiccants and/or application time of such chemicals, thus the current study is the first to explore this area. Figure 5.4. depicts the differences in thermal properties between soft dough and ripe application times for harvest samples.
Figure 5.4. DSC profiles for harvest samples treated with glyphosate at different levels of physiological maturity compared to the controls treated at the same stages

As shown in Figure 5.4, when comparing the thermal properties of harvest wheat samples treated at soft dough and ripe stage, the onset temperature and enthalpy did not show significant differences for application at the two stages. The end temperature was significant different for treatment, where glyphosate treated samples had a higher end temperature compared to the samples treated with water. Peak height, peak area and enthalpy were showed significant differences for application time, where the values were higher when application was performed at the soft dough stage. The interaction between treatment and application was not significantly different for any of the DSC parameters analyzed. According to Table 5.3, treatment did not causes significant differences for DSC parameters for the samples treated at soft dough stage or ripe stage. The difference could be because the harvest samples were analyzed not separately for soft dough and ripe (as the time series analysis), but together to compare soft dough treated samples and ripe stage treated samples. As reported by Xie et al. (2008), the gelatinization temperature represents the stability of the crystalline structure, which is determined by starch
granule size distribution. Thus, minor changes in the granule size distribution when comparing glyphosate and water and the two different application stages, might explain the differences in the thermal properties of the harvested samples. As explained, many characteristics such as, peak height, peak area and enthalpy were affected by application time, which shows that the stage of physiological development when the plant comes into contact with glyphosate is important in determining starch thermal properties. Additionally, Singh et al. (2010) determined that starch with higher crystallinity have higher enthalpy. Therefore, increased enthalpy at soft dough stage could be due to changes in the crystalline properties of starch caused by early application. When considering starch thermal characteristics and wheat end-use quality, Eliasson et al. (1995) determined that differences in thermal characteristics (analyzed using DSC) may not have significant impacts on the baking quality of flour. Thus, the differences observed in the harvest samples may not cause significant changes to end-use quality.

5.4.5. Amylopectin chain length distribution

Gel permeation chromatography was used to separate the amylopectin and amylose fractions from extracted wheat starch as explained earlier. The phenol-sulfuric assay and the blue value assays were used to identify these fractions, and the amylopectin fraction was used to determine the chain length distribution. Figure 5.5 shows the chromatogram corresponding to the separation of amylopectin and amylose using gel permeation chromatography followed by the phenol sulfuric assay. The peak that elutes first corresponds to the amylopectin fraction, which has higher molecular weight compared to the amylose fraction.
Figure 5.5. Low-pressure gel permeation column separation of amylopectin and amylose. Large amylopectin molecules are eluted first followed by amylose.

The method for amylopectin chain length analysis separated the different chains of different DPs elute as separate peaks. As example of such a chromatogram is shown in Figure 5.6. Table 5.4 shows the results of the amylopectin chain length analysis.

Figure 5.6. Amylopectin chain length analysis using high performance anion exchange chromatography (HPAEC): Example chromatogram. The different peaks shown correspond to different DPs, ranging from DP 6 to ≥ 40.
Table 5.4. Effect of glyphosate on amylopectin chain length

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time point</th>
<th>DP6 - 12</th>
<th>DP13 - 24</th>
<th>DP25 - 36</th>
<th>DP37-40</th>
<th>Average chain length (weight averaged)</th>
<th>Average chain length (number averaged)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Soft dough</td>
<td>Ripe</td>
<td>Soft dough</td>
<td>Ripe</td>
<td>Soft dough</td>
<td>Ripe</td>
</tr>
<tr>
<td>Glyphosate</td>
<td>T0</td>
<td>31.22 a</td>
<td>30.92 a</td>
<td>55.84 a</td>
<td>56.18 b</td>
<td>11.93 a</td>
<td>11.92 b</td>
</tr>
<tr>
<td></td>
<td>Tm</td>
<td>27.16 b</td>
<td>27.19 a</td>
<td>59.02 a</td>
<td>59.02 a</td>
<td>12.93 a</td>
<td>12.89 b</td>
</tr>
<tr>
<td></td>
<td>Te</td>
<td>27.54 a</td>
<td>27.72 a</td>
<td>58.68 b</td>
<td>58.65 b</td>
<td>12.84 a</td>
<td>12.73 b</td>
</tr>
<tr>
<td>Water/</td>
<td>T0</td>
<td>29.18 b</td>
<td>26.63 b</td>
<td>57.57 b</td>
<td>59.05 a</td>
<td>12.37 a</td>
<td>13.40 a</td>
</tr>
<tr>
<td>control</td>
<td>Tm</td>
<td>28.99 a</td>
<td>26.89 b</td>
<td>57.73 b</td>
<td>59.14 a</td>
<td>12.40 a</td>
<td>13.07 a</td>
</tr>
<tr>
<td></td>
<td>Te</td>
<td>26.29 b</td>
<td>26.77 b</td>
<td>59.89 a</td>
<td>58.98 a</td>
<td>12.96 a</td>
<td>13.33 a</td>
</tr>
<tr>
<td>Overall</td>
<td>Glyphosate</td>
<td>28.64 a</td>
<td>28.61 a</td>
<td>57.85 a</td>
<td>57.95 b</td>
<td>12.56 a</td>
<td>12.51 b</td>
</tr>
<tr>
<td></td>
<td>Water/</td>
<td>28.15 b</td>
<td>26.77 b</td>
<td>58.40 b</td>
<td>59.06 a</td>
<td>12.58 a</td>
<td>13.27 a</td>
</tr>
<tr>
<td>control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*aFor the time series data, the LSD values show if there is a significant difference at each time point for the treatment vs. control (T0 glyphosate vs. T0 control).
As shown in Table 5.4, glyphosate application at soft dough stage had a significant effect on DP6-12, DP13 - 24 and DP37 – 40, where glyphosate treated samples had higher percentage of chains of these chain lengths. Glyphosate application at soft dough stage significantly decreased the average chain length when considering number averaged chain length, however did not show any significant differences for weight averaged chain length. Glyphosate application at ripe stage caused significant differences in the proportion of the short and medium chain amylopectin molecules (DP6 – DP36) when compared to the control. The proportion of chains in the DP6-12 group was higher in the glyphosate treated samples compared to the control, and vice versa for DP13 – 24 and DP25 – 36. Additionally, for the ripe stage application, the average chain length was higher in the control compared to the glyphosate treated samples, which is similar to the observation for soft dough stage application.

When considering the proportion of amylopectin chains in the DP 6 – 12 category at all time points, in general the glyphosate treated samples had higher proportions compared to the control for both soft dough and ripe treatments. For DP13 – 24, in general the glyphosate treated samples at soft dough stage had higher amounts compared to the control at T₀ and T_mid, whereas for the ripe application stage it was vice-versa. For DP 25 – 36, the soft dough treated samples did not show significant differences compared to the control at all timepoints, whereas for ripe treatment the glyphosate treated samples showed lower amounts compared to the control at all times. For DP37 – 40, for soft dough stage, the treated samples showed higher percentage at T₀ and T_end, whereas for ripe stage application significant differences were only found at T₀, where the control showed significantly higher amounts. The average chain length was lower in the ripe treated samples compared to the control at all time points. A similar trend was observed for most time points for the soft dough treated samples as well. Overall, these results suggest that the pre-
harvest application of glyphosate has a significant effect on the proportion of amylopectin molecules with different chain lengths, especially for short and medium chain amylopectin. Application at both stages appear to decrease the average amylopectin chain length. Soft dough stage application appears to increase the percentage of DP6-12, DP13-24 and DP25-36 categories compared to the control, whereas ripe stage application has the opposite effect on DP13-24 and DP25-36. Therefore, the data suggests that application at soft dough or ripe gives varying results. Additionally, the results also showed that amylopectin chains of DP6-12 and DP37-40 decreased in proportion over time, whereas chains of DP13-24 and DP25-36 increased. The average amylopectin chain length also showed an increasing trend. These observations show how amylopectin chain length characteristics change during the physiological maturation of wheat.

In a review by Bertoft (2017) the average chain length of amylopectin (number averaged) is reported as 17.7. In the present study, the number averages chain length ranged from 14.37 to 15.08, which is somewhat lower than that of Bertoft (2017). Jane et al. (1999) and Franco et al. (2002) used a similar method to determine amylopectin chain length of wheat starch and reported the averages for DP6-12, DP13-2 and DP-25-36 as approximately 19, 42, and 16. For ≥37, they reported 13 and 20 respectively. The values obtained in the current study are 28.0, 58.3, 12.7 and 0.9, which are different than that of Jane et al. (1999) and Franco et al. (2002), which could be due to reasons such as type of wheat used in the study (hard red spring wheat), genotype differences and environmental conditions. In a different study, Hanashiro et al. (1996) determined the relative area of the different DP categories as 27%, 49%, 14% and 10%, which is somewhat in agreement with the results of the current study for DP6-12 and DP25-36. Additionally, Singh et al. (2010) found that the distribution of wheat amylopectin chain length
could vary between 44.5-52.4%, 43.8-50.5% and 3.7-6.5% for DP6-12, DP13-24 and DP≥24 respectively, while Singh et al. (2009) reported the distribution for the same categories as approximately 45%, 50% and 6%, highlighting the wide variation for amylopectin chain length distribution in different wheat cultivars under different conditions.

Figure 5.7. Differences in amylopectin chain length for chains of DP 6-12 (A), and overall amylopectin chain length average (B)

When considering the differences in amylopectin chain length characteristics in the harvest samples, significant differences were found between the glyphosate treated and the control samples for average amylopectin chain length (number averaged) as shown in Figure 5.7-B. Additionally, the proportion of DP6-12 chains was significantly different for treatments, application time and the interaction of these two factors. For treatment, as shown in Figure 5.7-A, the percentage was higher in the glyphosate treated samples compared to the control. Additionally, the percentage of DP37-40 amylopectin chains was significant different for the treatment and application time interaction. Treatment was also significant for the DP13-24 chain length. Overall, the results of the amylopectin chain length analysis shows that glyphosate application (regardless of time of application) reduced the average chain length, and time series
analysis showed that application at ripe or soft dough had varying effects on short and medium length amylopectin chains.

5.5. Conclusions

The goal of this study was to determine the effect of glyphosate application time on wheat starch chemical properties. A greenhouse study was conducted for this purpose, and glyphosate was applied at the recommended rate at soft dough stage and ripe stage, during which water was also sprayed on the control samples. Wheat heads were collected prior to application and every three days until harvest. Microscopy techniques were used to determine the spatial distribution of starch granules and morphological changes. The whole wheat flour samples were analyzed for amylose and amylopectin percentage and molecular weight. Starch extracted from the samples were analyzed for differences in thermal properties using differential scanning calorimetry and amylopectin chain length. The spatial distribution of starch granules, granule morphology, and amylose and amylopectin molecular weight were not affected by the glyphosate treatment. The thermal properties (analyzed using DSC) of harvest samples showed that glyphosate treated samples have higher end temperature, and that application at soft dough stage causes increases in peak height, peak area and enthalpy. However, amylopectin chain length showed some differences, where glyphosate application decreased the average amylopectin chain length. Furthermore, application at soft dough or ripe stage also affected the proportion of short and medium length amylopectin. The differences in chain length could be caused by glyphosate’s effect on starch deposition pathways. Application of glyphosate, which leads to an accumulation of shikimic acid has been shown to interrupt the carbon flow of affected plants, thus, starch biosynthetic pathways may also be impacted. Additionally, glyphosate’s metal chelation ability may cause changes to the activity of enzymes involved in the starch
biosynthesis. Thus, this study shows that glyphosate has some effect on biosynthetic pathways in plants, although the exact mechanisms and interactions responsible for such outcomes are unclear. Therefore, further studies are needed to firmly establish the effects of glyphosate on plant biosynthetic pathways, possibly using many different wheat cultivars, different application rates, and different environmental conditions as these factors affect the physiological development, hence the physicochemical properties of wheat. Thus, the interaction of these factors and glyphosate application can cause significant changes to the chemistry of wheat starch.

5.6. References


6.1.  **Abstract**

Some herbicides, such as glyphosate (N-phosphonomethyl glycine), are used as pre-harvest desiccants during wheat cultivation. Glyphosate inhibits the enolpyruvylshikimate phosphate synthase enzyme in the shikimic acid pathway. Humans do not share this biosynthetic pathway, however it is found in human gut microbiota. In this context, the aim of this exploratory study was to determine if there is an interaction between glyphosate and gut microbiota. A field study was performed, where glyphosate was sprayed at 1.1 kg of active ingredient/ha at soft dough and ripe stages of development, and harvested upon maturity. A control without glyphosate application was also included. The whole wheat flour samples were subjected to *in vitro* fecal fermentation using two donor fecal sample, after which short chain fatty acids (SCFA), ammonia, and microbiota composition were evaluated. In a second experiment, glyphosate was spiked at levels of 10, 20 and 30 ppm, to a base wheat flour sample assumed to be free of glyphosate, which was subjected to *in vitro* digestion, after which the same analyses mentioned above were performed. Significant differences (*P* ≤ 0.05) were not found for SCFA and ammonia production between the three treatments in both experiments. However, fecal donor appeared to be a significant factor impacting SCFA and ammonia production. The interaction between treatment and donor was significant for acetate in the spiking experiment. In the microbiota composition analysis, most genera did not show significant differences (*P* ≤ 0.05) among treatments, although the abundance of some genera decreased after fermentation. In particular, a general decrease in the relative abundance of *Clostridium* species was observed with increase in glyphosate levels. Overall, this study suggests that glyphosate does not have a
profound impact on gut microbiota metabolism, although glyphosate showed an inhibitory effect on the commensal *Clostridium* genus. Furthermore, a significant host x treatment interaction suggested that the impact of glyphosate on the gut microbiota may not be consistent among individuals.

6.2. Introduction

The human gut microbiota can be described as a “collection of bacteria, archaea and eukarya colonizing the human body” (Thursby and Juge 2017). These microorganisms have evolved over thousands of years and have created a mutually beneficial relationship with the host (Backhed et al. 2005). Some studies have suggested that there are ten times as many bacterial cells compared to human cells. However, recent findings show that the ratio between human and bacterial cells is close to 1:1 (Sender et al. 2016). Examples of gut bacteria include, *Ruminococcus, Clostridium, Lactobacillus, Eubacterium, Faecalibacterium* and *Roseburia* which belong to the Firmicutes phylum, and genera such as *Prevotella* and *Xylanibacter*, which belong to the Bacteroidetes phylum (Tremaroli and Backhed 2012). Additionally, *Collinsella* and *Bifidobacterium* genera belong to the Actinobacteria phylum. The gut microbiota perform a wide range of beneficial functions such as deriving energy from host dietary components, nutrient metabolism, xenobiotic and drug metabolism, antimicrobial functions, immunomodulation and maintenance of the gut integrity (Jandhyala et al. 2015).

Although the mode of action of glyphosate is exclusive to higher plants, algae, bacteria and fungi, the shikimic acid pathway, which is targeted by glyphosate, is also found in gut microbiota (Littman and Pamer 2011). The 5-enolpyruvyl shikimate 3-phosphate synthase (EPSPS) enzyme in the shikimic acid pathway is inhibited by glyphosate, which disrupts the production of aromatic amino acids such as, tyrosine, tryptophan and phenylalanine. The
inhibitory action of glyphosate is caused by chelating manganese, which is needed for the reduction of flavin mononucleotide, which acts as a co-factor for EPSPS. Some studies have suggested that glyphosate can spread through the ecosystem and has been detected in green immature seed (Lorenzatti et al. 2004), harvested seeds (Simonetti et al. 2015) and in ground water (Sanchis et al. 2012). Additionally, there have been some studies have discussing the possibility of interactions between gut microbiota and livestock.

In this context, Shehata et al. (2013) studied interactions between glyphosate and beneficial gut microbiota in poultry, and found that highly pathogenic microorganisms such as, *Salmonella enteritidis, Salmonella gallinarum, Salmonella typhimurium, Clostridium perfringens* and *Clostridium botulinum* have high resistance to glyphosate, whereas beneficial bacteria such as, *Enterococcus faecalis, Enterococcus faecium, Bacillus badius, Bifidobacterium adolescentis* and *Lactobacillus* sp. are susceptible. Thus, it was concluded that decreases in the beneficial gut bacterial populations in poultry could lead to imbalances in the normal gut bacterial community. Additionally, the results suggested that glyphosate’s inhibitory effects on *Enterococcus* sp. could be a reason for the increased incidence of *C. botulinum*- associated disease, due to the suppression of the antagonistic effects of *Enterococcus* sp. on Clostridia. Similarly, Ackermann et al. (2015) studied the impact of glyphosate on microbiota and botulinum neurotoxin expression during ruminal fermentation and found that glyphosate has inhibitory effects on selected groups of ruminal microbiota, whereas it caused an increase in the population of pathogenic bacteria. Moreover, Kruger et al. (2013) also investigated interactions between cattle gut microbiota and glyphosate, and possible effects on increase of *Clostridium botulinum* associated diseases and found that glyphosate inhibits the antagonistic effects of *Enterococcus* sp. on *Clostridium botulinum* leading to increased infections caused by the latter. Thus, the study
concluded that glyphosate causes dysbiosis, which ultimately favors the production of botulinum neurotoxin. On the other hand, Riede et al. (2016) did not find any adverse effects on ruminal metabolism which could be caused by glyphosate, especially on the growth of Clostridia.

Glyphosate’s toxic effects on aquatic species have also been studied. For example, Kittle et al. (2018) studied interactions between glyphosate and the gut microflora of Hawaiian green turtles (Chelonia mydas), because the coastal areas near Hilo, Hawaii because this area is subjected to flash flooding and run off, which could lead to glyphosate reaching aquatic organisms. For this purpose, an in vitro study was performed, where bacterial samples from gastrointestinal tracts of green turtles was exposed to 6 different concentrations of glyphosate for a period of 24 hours, after which bacterial density was determined. The results of the study indicated that bacterial isolates such as, Pantoea, Proteus, Shigella, and Staphylococcus are inhibited by glyphosate at concentrations of more than 1.76 x 10^{-3} g/L, thus leading to detrimental effects on digestion and overall health of green turtles. On a different note, Nielsen et al. (2018) found that glyphosate has very limited effects on microflora of Sprague Dawley rats during a two week exposure trial. The study also indicated that the effect of glyphosate is dependent on the availability of aromatic amino acids, which could mean that the observed effects was influenced by the availability of aromatic amino acids in the gastrointestinal environments of the experimental rats. Additionally, the study found that there is a strong correlation between glyphosate and intestinal pH, which could be associated with reductions in the production of acetic acid. Thus, it was concluded that sufficient diet derived aromatic amino acids alleviates the need for production of such amino acids by gut bacteria, which could prevent the antimicrobial effects of glyphosate in the gut. Additionally, recent studies have also indicated that glyphosate is cytotoxic to different cells in in vitro studies (Benachour and Seralini 2009;
Benachour et al. 2007). Moreover, adjuvants used in glyphosate formulations is thought to increase its toxic effects.

In this context, the objective of this study was to use in vitro fecal fermentation studies to determine if there are interactions between glyphosate and human gut microbiota. For this purpose, whole wheat samples were digested in vitro and used in fermentation studies with different levels of glyphosate.

6.3. Materials and methods

6.3.1. Materials

A field study was conducted where glyphosate was applied at a rate of 1.1 kg of active ingredient/ha at the soft dough and ripe stages of physiological development. A control in which glyphosate was not applied was also used in the study. This study was performed with two hard red spring wheat cultivars (Glenn and Prosper), at three locations in North Dakota (Carrington, Minot and Prosper). Each treatment was repeated three times resulting in 54 total samples. This 3 x 3 x 2 (location x treatment x cultivar) factorial design was used with a split-plot lay out, with location as the main plot, and treatment x cultivar as the subplot.

6.3.2. Compositional analysis

The harvested samples were milled in an in-house milling facility using a Buhler-202 mill. The whole wheat samples from the field study were combined to 18 samples, which were analyzed for moisture (AACCI method 44-15.02), protein (AACCI method 46-30.01) and starch (AACCI method 76-13.01).

6.3.3. In vitro digestion of whole wheat samples

The in vitro digestion was performed as previously described by Yang and Rose (2014). A volume of 300 mL of water was added to 25g of sample, and boiled for 20 min, stirring
constantly to avoid the formation of lumps. The samples were allowed to cool down, and the pH was adjusted to 2.5 with 1M HCl. Then 10 mL of 10% pepsin in 50 mM HCl was added, and the samples were kept in an orbital shaker at 150 rpm and 37°C for 30 min. Afterwards, 50 mL of 0.1 maleate buffer (pH 6, containing 50 mM CaCl₂) was added and the pH of the samples was adjusted to 6.9 with 1 M NaHCO₃. Then 50 mL of 12.5% pancreatin in maleate buffer and 2 mL amylglucosidase (3260 U/mL, Megazyme, Bray, Ireland) were added, and the samples were kept in a shaking water bath at 150 rpm and 37°C for 6 hours. The digested samples were then dialyzed for 3 days at 4°C in 3500 molecular weight cut off dialysis tubing (Spectrum Laboratories, Rancho Dominguez, CA, U.S.A). The water was changed every 3 hours during the day when dialysis was performed. The samples were then freeze dried, and compositional analysis was performed again as described in section 6.3.2.

### 6.3.4. In vitro fecal fermentation

Fecal fermentation was performed after Yang and Rose (2014). An amount of 15 mg of digested, freeze dried sample was suspended in 1 mL of sterile fermentation medium and left over night to hydrate. The next day, the fecal slurry was prepared by mixing fecal samples and sterile phosphate buffer (PBS, pH 7.0), in a ratio of 1:9 (w/v) using a hand blender for 1 min, which were then filtered through four layers of cheese cloth. During this experiment, two fecal samples were used, and each treatment had two duplicates. The samples were then inoculated with 0.1 mL of fecal slurry, and incubated in an orbital shaker at 125 rpm, at 37°C for 12 hours. Samples were collected at 0 hour and 12 hour, and immediately frozen with liquid nitrogen and placed at -80°C. The fermentation steps were performed in an anaerobic hood with 5% H₂, 5% CO₂ and 90% N₂.
A spiking study was also conducted using a control sample from the field study which was assumed to be free of glyphosate. To the digested sample, glyphosate was spiked at levels of 10, 20 and 30 ppm, and like for the field samples, spiked samples were fermented with two fecal donor samples, and samples were collected at 0 and 12 hours. Metabolite analysis was performed according to the same method for both field samples and spiked samples.

6.3.5. Metabolite analyses

S/BCFA and ammonia analyses were performed after Yang and Rose (2014). In short, 1 mL of sample, 0.5 mL of internal standard, 0.25 mL of 9M sulfuric acid and 0.16 g of NaCl were mixed together. Then 0.5 mL of diethyl ether was added and shaken for approximately 1 min. Once the layers were separated, the diethyl layer was transferred to a centrifuge tube containing 0.02 mL of 2M NaOH. The sample was re-extracted in the same manner. Then 1 μL of the diethyl ether layer was injected to the GC. The amount of ammonia in the samples was quantified using the method the phenol-chloroform method (Solórzano 1969), with adaptations (Yang and Rose, 2014).

6.3.6. Microbiota composition

Microbiota compositional analysis was performed on the spiked samples. DNA isolation was performed according to the method of Martinez et al. (2009), where mechanical and enzymatic cell lysis were used together with a subsequent phenol/chloroform extraction. Samples were sequenced on the Illumina MiSeq platform for 16S rRNA paired-end sequencing following the protocol of Kozich et al. (2013). Sequences were demultiplexed and barcodes were removed prior to sequence analysis. Sequence quality was checked using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and reads were trimmed to 240 bp with the FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). Sequences with a phred score
of less than 20 were trimmed using TrimGalore (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) and paired-ends were merged with PANDAseq (Masella et al. 2012) with a threshold score of 0.9. Operational taxonomic units (OTUs) were generated at 97% similarity using subsampled open reference OTU picking in QIIME (Caporaso et al. 2010) and taxonomy was assigned.

6.3.7. Data analysis

The data from the compositional analysis of the whole wheat flour samples and the digested samples was analyzed using the SAS software (version 9.3, SAS Institute, Cary, NC). To analyze the fermentation outcomes data (S/BCFA, ammonia and fermented carbohydrates) the difference of metabolites between T12 and T0 was taken into consideration. The same method was used to analyze the data from the microbiota composition analysis.

6.4. Results and discussion

6.4.1. Compositional analysis of whole wheat samples

The whole wheat flour samples and digested flour samples were analyzed for moisture, protein, starch and dietary fiber content as explained in the methods. These results are summarized in Table 6.1. The moisture content of whole wheat samples before and after in vitro digestion appeared to be similar. However, the protein content increased after digestion, whereas the starch content decreased. The general trends for starch and protein are in agreement with those of Brahma et al. (2017). Additionally, the starch and protein contents in the whole wheat samples used in this study are similar to the values reported for whole wheat flour by Brahma et al. (2017). The values obtained for starch content of digested whole wheat flour is in agreement with the findings of Englyst et al. (2007) who reported that whole meal bread contains approximately 1.9% resistant starch.
Table 6.1. Summary of composition analysis\textsuperscript{a}

<table>
<thead>
<tr>
<th>Sample</th>
<th>Moisture (%)</th>
<th>Protein (%)</th>
<th>Starch (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before digestion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>12.6 ± 0.3</td>
<td>14.2 ± 1.6</td>
<td>54.4 ± 0.9</td>
</tr>
<tr>
<td>Ripe</td>
<td>12.6 ± 0.2</td>
<td>14.2 ± 1.6</td>
<td>54.0 ± 0.9</td>
</tr>
<tr>
<td>Soft dough</td>
<td>12.6 ± 0.2</td>
<td>13.9 ± 1.5</td>
<td>53.5 ± 0.9</td>
</tr>
<tr>
<td>After digestion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>11.8 ± 0.9</td>
<td>21.7 ± 2.2</td>
<td>1.4 ± 0.7</td>
</tr>
<tr>
<td>Ripe</td>
<td>11.4 ± 0.8</td>
<td>22.5 ± 5.3</td>
<td>0.9 ± 0.5</td>
</tr>
<tr>
<td>Soft dough</td>
<td>11.8 ± 0.5</td>
<td>22.0 ± 5.0</td>
<td>1.1 ± 0.7</td>
</tr>
</tbody>
</table>

\textsuperscript{a}The results presented in the table are averaged across cultivars and location.

6.4.2. Analysis of S/BFA as measured of the metabolism of gut microbiota

Short/branched chain fatty acids and ammonia content before and after fermentation was determined as a measure of metabolite production by gut microorganisms. The difference in the amount of fatty acids produced at 0 and 12 hr was used to determine the effects of treatment, location, cultivar, fecal donor and the different interactions. Table 6.2 summarizes these results.

Table 6.2. Summary of metabolite analysis in field samples\textsuperscript{a}

<table>
<thead>
<tr>
<th>Effect</th>
<th>Acetate (mM)</th>
<th>Propionate (mM)</th>
<th>Butyrate (mM)</th>
<th>Iso-butyrate (mM)</th>
<th>Iso-valerate (mM)</th>
<th>Ammonia (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment\textsuperscript{b}</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Con</td>
<td>24.39 a</td>
<td>7.07 a</td>
<td>4.99 a</td>
<td>0.12 a</td>
<td>0.09 a</td>
<td>52.04 a</td>
</tr>
<tr>
<td>RP</td>
<td>23.34 a</td>
<td>7.02 a</td>
<td>4.23 b</td>
<td>0.10 a</td>
<td>0.72 a</td>
<td>49.31 b</td>
</tr>
<tr>
<td>SD</td>
<td>24.57 a</td>
<td>6.90 a</td>
<td>5.07 a</td>
<td>0.09 a</td>
<td>0.90 a</td>
<td>51.75 a</td>
</tr>
<tr>
<td>Fecal donor</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Donor I</td>
<td>22.53 b</td>
<td>6.68 a</td>
<td>5.08 a</td>
<td>0.06 b</td>
<td>0.42 a</td>
<td>50.12 b</td>
</tr>
<tr>
<td>Donor II</td>
<td>25.66 a</td>
<td>7.31 a</td>
<td>4.44 b</td>
<td>0.15 a</td>
<td>0.72 a</td>
<td>51.95 a</td>
</tr>
<tr>
<td>Location\textsuperscript{c}</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Car</td>
<td>25.59 a</td>
<td>6.78 a</td>
<td>5.18 a</td>
<td>0.09 a</td>
<td>1.63 a</td>
<td>50.29 b</td>
</tr>
<tr>
<td>Min</td>
<td>23.24 a</td>
<td>7.13 a</td>
<td>4.65 b</td>
<td>0.11 a</td>
<td>0.09 a</td>
<td>50.59 b</td>
</tr>
<tr>
<td>Pros</td>
<td>23.47 a</td>
<td>7.08 a</td>
<td>4.45 b</td>
<td>0.12 a</td>
<td>0.16 a</td>
<td>52.23 a</td>
</tr>
<tr>
<td>Cultivar</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glenn</td>
<td>23.82 a</td>
<td>6.93 a</td>
<td>4.74 a</td>
<td>0.11 a</td>
<td>0.42 a</td>
<td>51.11 a</td>
</tr>
<tr>
<td>Prosper</td>
<td>24.38 a</td>
<td>7.06 a</td>
<td>4.78 a</td>
<td>0.10 a</td>
<td>0.72 a</td>
<td>50.95 a</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Means with the same letter are not significantly different at the $P \leq 0.05$ significance level.

\textsuperscript{b}Con: Control, RP: Ripe, SD: soft dough.

\textsuperscript{c}Location – Car: Carrington, Min: Minot, Pros: Prosper.
As shown in Table 6.2, considering short chain fatty acids, only butyrate showed significant differences for treatment, where the level was highest at the soft dough treatment. The SCFA produced by gut microbiota are important in physiological functions, for example they are oxidized by mucosal cells to provide energy (Macfarlane and Macfarlane 2012). Additionally, butyrate, is important in regulating cell growth, differentiation, cytoskeleton organization and modulation of gene expression. In fact, the order of significance of SCFA can be ranked as butyrate > propionate > acetate. When considering donor specific results for butyrate, for Donor II, the level of butyrate was lower in the treated samples compared to the control. However, this was not the case for Donor I. Therefore, overall the results seem to indicate that the effect of butyrate production may be impacted to different degrees depending on the individual.

Moreover, according to Macfarlane and Macfarlane (2012) bacterial genera such as *Roseburia, Faecalibacteria, Clostridia, Eubacteria, Fusobacteria, Peptostreptococci, Butyribrio* and *Peptococci* produce butyrate by fermentation. Thus, the population of these genera could be different between individuals leading to different responses when exposed to glyphosate.

Branched chain fatty acids did not show significant differences for treatment. These fatty acids are reduced carbon skeletons of amino acids such as valine and leucine, which form iso-butyrate and iso-valerate respectively (Macfarlane and Macfarlane 2012). Clostridia species form large amounts of BCFA as determined by Elsdon and Hilton (1978). Since BCFA originate from amino acids, the production of these fatty acids can be considered as an indicator for protein digestion in the large intestine. Significant differences were also seen for ammonia production, where the highest values was found in the control and the lowest in the ripe treated samples. However, when considering the results of the two donors separately, Donor II showed the highest amount of ammonia for the soft dough treatment. The products of protein metabolism
(BCFA and ammonia) could be utilized for cellular processed if saccharolytic bacteria are present (Macfarlane and Macfarlane 2012), however, the accumulation of these products have also been linked to adverse effects on human health, and have been associated with increased risk of colorectal cancer and ulcerative colitis (Flickinger et al. 2003). When considering the results for metabolite analysis, it appears that glyphosate application time may not have a significant impact on the production of S/BCFA and ammonia by human gut microbiota since a clear trend in terms of application time and metabolite production was not observed. However, as Morrison and Preston (2016) explained in their review article, different bacterial phyla are responsible for producing specific S/BCFA as exemplified above. Thus, glyphosate may affect these phyla differently. For example, as the article points out, acetate production is “widely distributed among bacterial groups” whereas the production of other metabolites such as propionate and lactate is “more highly conserved and substrate specific”. Additionally, as explained below, the contribution of the donor/host gut microbiota population also plays a significant role in determining the effect of glyphosate on gut microorganisms.

As many previous studies have determined, fecal donor dietary pattern is a major factor affecting the functionality and metabolite production of gut microorganisms. For example, Brahma et al. (2017) determined that dietary pattern of fecal donor affects in vitro fermentation of whole grains and bran and Yang and Rose (2014) reported that dietary pattern of fecal donor affects butyrate production. In the current study, two fecal donors were used to determine the effect of glyphosate timing on wheat desiccation on human gut microbiota. As Table 6.2 shows, except propionate and iso-valerate production, fecal donor caused significant differences in the level of S/BCFA. Donor II showed significantly higher levels of acetate, iso-butyrate and ammonia, and lower levels of butyrate. Previous work on gut microbiota and diet have shown
that a diet rich in plant fiber components is beneficial for maintaining gut health (Martínez et al. 2012). Therefore, differences observed in the production of microbial metabolites with respect to fecal donor is possibly due to differences in the dietary pattern of fecal donors which leads to differences in the dynamics of his/her gut microbiota. Moreover, the results highlight the importance of the donor gut microbiota population and how it affects the interference of glyphosate on the metabolic reactions of these organisms.

As explained in the methods, the present study was conducted in three different environments. Significant differences were found for butyrate, iso-valerate and ammonia production concerning location. The amount of herbicides that crops come into contact with could vary depending on drift effects from surrounding areas (Brain et al. 2017; Gove et al. 2007; Marrs et al. 1991; Marrs et al. 1989). Thus, differences found between locations could be due different levels of glyphosate exposure, thus accumulation, depending on drift exposure levels at the different locations used in this study. Additionally, herbicide carryover at different locations could be a contributing factor causing differences between locations (Grey et al. 2012). The current study did not show any significant differences between cultivars for metabolite production possibly due to the fact that dietary fiber components are found within certain ranges in specific crop species, and cultivar differences are usually not significant. For example De Santis et al. (2018) determined that total arabinoxylan content in old and modern durum wheat cultivars are not significantly different.

Interaction between location x cultivar was not significantly different for any of the metabolites, while the location x treatment and location x donor was significantly different for butyrate and ammonia. Additionally, the cultivar x treatment and the cultivar x donor interactions were not significantly different for all metabolites. The treatment x donor interaction
was only significantly different for ammonia production. The three-way interactions were not significant for most metabolites except ammonia and butyrate. This is due to the significant effects on treatment, location and donor explained above. As for the different interactions, the treatment x donor interaction is important as it shows that importance of the donor microbiota population and the effect it could have on metabolism of gut microbiota if such microorganisms come into contact with glyphosate. Additionally, as mentioned above, the interactions with location are possibly due to changes in the amount of glyphosate caused by drift effects and/or carry over effects, which highlight the importance of determining factors affecting the amount of glyphosate in crops not only due to direct spraying but also due to indirect exposure methods.

Figure 6.1 below shows the different trend for treatment for SCFA and ammonia for the different fecal donors.

![Figure 6.1. Effect of glyphosate timing and fecal donor on SCFA and ammonia production](image-url)

When considering the trend for SCFA production, both donors showed similar trends for the different treatments, where the highest level was seen in soft dough and lowest in ripe. This raises the question if glyphosate which could be a source of glycine or phosphate. Early application could potentially increase the accumulation of glyphosate in wheat which could result in higher amount of SCFA production. Late application at ripe stage may not cause such effects or have a negative impact leading to lowest levels of SCFA being produced during ripe stage.
application. As for ammonia production, Donor I showed the highest level in the control and the
lowest in ripe, whereas Donor II showed the highest in soft dough and lowest in ripe. These
differences may be due to changes in the gut microbial population of the two donors in this
study. Since previous studies on gut microbiota have not focused in these microorganism and
glyphosate, it is somewhat difficult to compare the results of the current study with similar work.
Previous work on Sprague rats and exposure to glyphosate found that the effect of glyphosate is
dependent on the availability of aromatic amino acids (Nielson et al. 2018). Thus, the findings of
this study are somewhat in agreement with these findings as glyphosate does not have profound
effect on the metabolism of gut bacteria in humans.

6.4.3. Analysis of microbial metabolites and fermented carbohydrates in spiked samples

Since the availability of glyphosate after the in vitro digestion procedure is not known, a
spiking study was also performed with glyphosate being spiked at levels of 10, 20 and 30 ppm to
a base flour (control from field study) to determine if similar observations to the field study can
be made. These levels of glyphosate were spiked because the EPA limit for glyphosate in wheat
is 30 ppm.
Table 6.3. Summary of microbial metabolite analysis of spiked samples

<table>
<thead>
<tr>
<th>Effect</th>
<th>Acetate</th>
<th>Propionate</th>
<th>Butyrate</th>
<th>Iso-butyrate</th>
<th>Iso-valerate</th>
<th>Ammonia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 ppm</td>
<td>23.18 a</td>
<td>7.47 a</td>
<td>4.87 a</td>
<td>0.11 a</td>
<td>0.05 a</td>
<td>50.07 a</td>
</tr>
<tr>
<td>20 ppm</td>
<td>23.13 a</td>
<td>7.62 a</td>
<td>4.80 a</td>
<td>0.12 a</td>
<td>0.03 a</td>
<td>51.75 a</td>
</tr>
<tr>
<td>30 ppm</td>
<td>25.54 a</td>
<td>7.98 a</td>
<td>5.27 a</td>
<td>0.12 a</td>
<td>0.07 a</td>
<td>51.04 a</td>
</tr>
<tr>
<td>Fecal donor</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Donor I</td>
<td>22.21 b</td>
<td>7.15 b</td>
<td>5.61 a</td>
<td>0.09 b</td>
<td>0.02 b</td>
<td>51.04 a</td>
</tr>
<tr>
<td>Donor II</td>
<td>25.68 a</td>
<td>8.24 a</td>
<td>4.35 b</td>
<td>0.15 a</td>
<td>0.12 a</td>
<td>50.86 a</td>
</tr>
<tr>
<td>Donor x Treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Donor I x 10 ppm</td>
<td>21.31 b</td>
<td>6.84 a</td>
<td>5.63 a</td>
<td>0.09 a</td>
<td>0.01 a</td>
<td>49.48 a</td>
</tr>
<tr>
<td>Donor I x 20 ppm</td>
<td>19.93 b</td>
<td>6.87 a</td>
<td>5.17 a</td>
<td>0.08 a</td>
<td>0.09 a</td>
<td>51.96 a</td>
</tr>
<tr>
<td>Donor I x 30 ppm</td>
<td>25.40 a</td>
<td>7.74 a</td>
<td>6.04 a</td>
<td>0.09 a</td>
<td>0.02 a</td>
<td>51.68 a</td>
</tr>
<tr>
<td>Donor II x 10 ppm</td>
<td>25.05 a</td>
<td>8.11 a</td>
<td>4.11 a</td>
<td>0.14 a</td>
<td>0.10 a</td>
<td>50.67 a</td>
</tr>
<tr>
<td>Donor II x 20 ppm</td>
<td>26.32 a</td>
<td>8.38 a</td>
<td>4.44 a</td>
<td>0.15 a</td>
<td>0.14 a</td>
<td>51.54 a</td>
</tr>
<tr>
<td>Donor II x 30 ppm</td>
<td>25.68 a</td>
<td>8.22 a</td>
<td>4.50 a</td>
<td>0.15 a</td>
<td>0.12 a</td>
<td>50.39 a</td>
</tr>
</tbody>
</table>

a Means with the same letter are not significantly different at the $P \leq 0.05$ significance level

In the spiking study, the level of S/BCFA was not significantly different for the different levels of glyphosate. However, in the field study with different glyphosate application times, butyrate and ammonia production was significantly impacted by glyphosate application time. The discrepancy between these two experiments could be due to glyphosate’s herbicidal action. That is, several studies have indicated that glyphosate affects the carbon flow of treated plants leading to negative impacts on photosynthesis. In this context, glyphosate could also affect production of non-starch polysaccharides, such as arabinoxylan, which is a major dietary fiber component in wheat. Therefore, further studies are needed to establish how glyphosate application time affects the chemistry of dietary fiber components in wheat. Additionally, when glyphosate is spiked into digested whole wheat samples, it may act as a metal chelator but will not cause any changes to the chemistry of wheat dietary fiber components. As in the field samples, fecal donor significantly affected the production of S/BCFA in this study too. As mentioned previously, dietary patterns of fecal donors have a major impact on the population of
gut microbiota, which could cause differences in gut fermentation outcomes. The interaction between treatment x donor was significant for acetate production, which further highlights the role of fecal donor gut microbiota population and the effect when gut microbiota is exposed to different levels of glyphosate. Acetate is produced by fermentative microorganisms like *Bacteroides, Bifidobacteria, Eubacteria, Lactobacilli, Clostridia* and *Ruminococci* (Macfarlane and Macfarlane 2012) and have been associated with adenosine triphosphate (ATP) generation (Macfarlane 1991). Thus, differences in the population of such microorganisms between the donors may have been a contributing factor to the observed differences. The effect on acetate production could also be due to effects of glyphosate and intestinal pH, as previously shown by Nielsen et al. (2018). When considering the increasing levels of glyphosate, the results did not show an inhibitory effect on the amount of acetate produced.

6.4.4. **Microbiota composition analysis in glyphosate spiked fermented samples**

As explained in the methods, 16S RNA sequencing methods were used to determine the effects of glyphosate on gut microbiota populations when glyphosate was spiked at levels of 10, 20 and 30 ppm to a base sample assumed to be free of glyphosate. Table 6.4 shows the mean abundances of different genera identified during the fermentation analysis. In the table, the most dominant genera are presented, which were identified by ranking the overall relative abundance at time point zero (averaged across treatments and donors).
Table 6.4. Relative abundance of dominant microbiota identified in the study\textsuperscript{a}

<table>
<thead>
<tr>
<th>Genus</th>
<th>Abundance</th>
<th>Effect of glyphosate treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>T=0 10 ppm</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ppm ppm ppm</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T=0 10 ppm</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ppm ppm ppm</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Genus</th>
<th>Abundance</th>
<th>Effect of glyphosate treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>T=0 10 ppm</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ppm ppm ppm</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T=0 10 ppm</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ppm ppm ppm</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Abundance data for each genus is presented under the columns for each concentration level (10 ppm, 20 ppm, and 30 ppm). The effect of glyphosate treatment (T=0 vs. T=12) is indicated by asterisks (*), with 
\textsuperscript{b} Values followed by an asterisk (*) indicate a significant change in abundance compared to the control group.
Table 6.4. Relative abundance of dominant microbiota identified in the study\(^{a}\) (continued)

<table>
<thead>
<tr>
<th>Genus</th>
<th>Abundance</th>
<th>Effect of glyphosate treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>T=0</td>
</tr>
<tr>
<td></td>
<td>DI</td>
<td>DII</td>
</tr>
<tr>
<td>Roseburia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slackia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leuconostoc</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adlercreutzia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bulleidia</td>
<td></td>
<td></td>
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\(^{a}\)The relative abundances reported for Donor I (DI) and II (DII) were averaged across treatments for T=0. Significant differences between treatments are indicated with the a, b or c for T=0 and T=12 separately. Significantly higher values for relative abundance at T=12 are indicated by * in the T=12 column, and significantly lower values at T=12 are indicated with * in the T=0 column. The relative abundance for each genus is shaded to indicate low to high amounts (light to darker color).

The dominant genera identified in this experiment belonged to the phyla Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria. One genus each of phyla Verrucomicrobia and Euryarchaeota were also identified. Previous work on gut microbiota identified 12 prominent phyla in the GI tract, and almost 95% of the species identified were categorized into the above mentioned phyla (Hugon et al. 2015). In previous work Brahma et al (2017), most genera identified in the current study were found in 16S RNA sequencing in relation of fecal fermentation studies. Therefore, the bacterial phyla identified in this study are in agreement with previous findings.
When considering the relative abundance of the different genera at T=0, significant differences were not identified for the dominant genera between Donor I and II as shown in Table 6.4. Therefore, both participants of this study had similar gut microbiota dynamics considering the most dominant genera identified earlier. Bacterial genera *Blautia*, *Faecalibacterium* and *Ruminococcus* were the most abundant genera, while other genera such as *Parvomonas* and *Lactobacillus* were less prominent. Similar observations were made for the different glyphosate treatments before fermentation (T=0). Following fermentation, the relative abundance on most of the genera identified showed significant decreases in comparison to T=0. However, some genera such as *Ruminococcus*, *Phascolarctobacterium* and *Collinsella* showed higher values for relative abundance at T=12, indicating that there was an increase in the population of the bacterial genera during the fermentation process. Brahma et al. (2017) also reported increases in the relative abundance of *Dorea*, *Bacteroides* and *Collinsella* in a study with different whole grains. In this study, similar observations were made for *Dorea* and *Collinsella* at all three levels of glyphosate treatment, however, *Bacteroides* showed lower relative abundance at 30 ppm, although at 10 and 20 ppm there was an increase in relative abundance when comparing T=12 with T=0. However, of these three genera, statistically significant increases were only observed for *Collinsella*. Additionally, genera such as, *Ruminococcus* (in family Lachnospiraceae), *Bifidobacterium* and *Adlercreutzia* also showed statistically higher abundance at T=12.

Bacterial genera such as *Blautia*, *Faecalibacterium*, *Ruminococcus* (in family Ruminococcaceae), *Streptococcus*, *Parabacteroides*, *Clostridium* and many others showed decreased abundance at T=12 compared to T=0. Similar observations were made for *Blautia* and *Faecalibacterium* by Brahma et al. (2017) during fermentation with whole grains. In this study,
statistically significant reductions were noted in *Faecalibacterium, Ruminococcus, Lachnospira, Oscillospira, Anaerostipes, Akkermansia, Turicibacter, Enterococcus, Clostridium, Roseburia, Leuconostoc* and *Dehalobacterium*. As shown in Table 6.4, several genera such as, *Enterococcus* and *Clostridium* showed significant reductions at higher levels of glyphosate, which indicates that higher levels cause inhibitory effects. In general, increasing glyphosate levels caused greater reductions in the relative abundance of genera, although these reductions were at times not significant different between the three different glyphosate levels. Krumbeck et al. (2015) determined that shifts in the composition of gut microbiota after fermentation could be due to various reasons, which include competition for nutrients and growth factors. However, the reason for this phenomenon has not been firmly established yet. As for the genus Clostridium, the different in relative abundance at T=12 and T=0 was significantly different between the different glyphosate treatments indicating increasing inhibitory effects as the concentration of glyphosate increased. Previous studies also found that glyphosate could have inhibitory effects of specific gut microorganisms in poultry (Shehata et al. 2013) and in cattle (Ackerman et al. 2015; Kruger et al. 2013). The sequencing results were also analyzed for correlations with metabolite production and these findings are presented in Table 6.5.
Table 6.5. Correlations between bacterial genera and metabolite productiona

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<tr>
<th>Genus</th>
<th>Acetate</th>
<th>Propionate</th>
<th>Butyrate</th>
<th>Isobutyrate</th>
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<td>Blautia</td>
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<td>Phascolarctobacterium</td>
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Color scale: 

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Significant correlations are indicated with *: \( P \leq 0.05 \), **: \( P \leq 0.01 \) and ***: \( P \leq 0.001 \). The color scale indicates positive or negative correlations as follows
In the correlation analysis, the difference in relative abundance between T=12 and T=0, was correlated with the difference in metabolite production (T12-T0). As shown in Table 6.5, several genera such as, *Coprococcus*, *Streptococcus*, *Lachnospira*, *Lactococcus*, *Catenibacterium*, *Bulleidia* and *Mogibacterium* showed significant positive correlations with the production or SCFA and BCFA. Additionally, *Lactobacillus* showed significant positive correlations with ammonia production. Significant negative correlations were found between *Dialister, Paraprevotella, Collinsella, Desulfovibrio, Butyricimonas* and *Mogibacterium* and S/BCFA production. According to Table 6.4, *Lachnospira* abundance decreased after fermentation, which may be caused by glyphosate’s antimicrobial action. Thus, at increasing levels of glyphosate, *Lachnospira* would be reduced which could lead to decreases in the production of metabolites. However, *Dialister* and *Collinsella* increased in abundance after fermentation, which could have opposite effects, as these genera showed significant negative correlations with S/BCFA production. Overall, the correlation analysis indicates that microbial metabolite production is favored by some microorganisms, while others can have negative effects. Thus, when analyzing the effect on glyphosate on microorganisms, it is important to distinguish which species are affected by glyphosate.

Although this study provides some evidence that glyphosate may cause some effects on the human gut microbiota depending on features of the host gut microbiome, further studies are required to firmly establish this association. As previously mentioned, Neilson et al. (2018) determined that the effects of glyphosate on human gut microbiota is dependent on the availability of aromatic amino acids in the gut environment, because the availability of such amino acids would compensate for the lack of aromatic amino acids caused by the inhibition of the shikimic acid pathway. Thus, *in vitro* studies in an environment without an abundant supply
of aromatic amino acids maybe required to understand how glyphosate interacts with human gut microbiota. Additionally, glyphosate’s antimicrobial activity could be due to its chemical properties as a metal chelator, which could lead to the unavailability of metal ions which are required for the enzyme catalyzed reactions. Nevertheless, these aspects should be taken into consideration when conducting further investigations in this area.

6.5. **Conclusions**

In this exploratory study, possible interactions between glyphosate and human gut microbiota were evaluated. Whole wheat samples from a field study where glyphosate was applied at the recommended stage (ripe), earlier than recommended (soft dough) and control where glyphosate was not applied were used in this study. After *in vitro* digestion and fermentation with two donor fecal samples (separately), microbial metabolites such as S/BCFA and ammonia were evaluated, and the results indicated that glyphosate may not have a profound effect on the metabolism of human gut microbiota as only butyrate showed some differences associated with treatment. However, there may be some effects which are also depended on the host dietary patterns and gut microbiota dynamics. Due to the difficulty of identifying the fate of glyphosate after ingestion, a second study was performed where analytical grade glyphosate was spiked at levels of 10, 20 and 30 ppm to a base flour sample (digested control flour sample from the field study) to determine the impact of glyphosate on microbial metabolites and gut microbiota populations. The results of the second experiment also indicated that the effect of glyphosate on human gut microbiota metabolism may not be significant since only acetate production appeared to be affected by the donor x treatment interaction. In the 16S RNA analysis, the results indicated that the relative abundance of bacterial genera were not significantly different between the donors. Furthermore, a decrease in abundance was observed
for most genera after fermentation, although the reductions observed were not significantly different between the different levels of glyphosate. Additionally, the results showed that glyphosate may have an inhibitory effect on *Clostridium* species, since greater decreases in relative abundance was observed at higher glyphosate concentrations. Thus, overall the results indicate that the effects of glyphosate on human gut microbiota may not be consistent among individuals indicating that the host gut microbiota plays a role in determining these effects, and that glyphosate may have inhibitory effects of some bacterial genera. To further improve this work, the fate on glyphosate in the human body can be firmly established and more extensive work can be carried out with multiple fecal donors with different dietary patterns and gut microbiota dynamics to understand the antimicrobial effects glyphosate may exert on human gut microbiota.

### 6.6 Acknowledgements

The authors would like to thank the North Dakota State University Hard Red Spring Wheat Breeding Program and Dr. Joel Ransom for providing the field samples. Members of the Cereal Grains Research Group at the Food Innovation Center, University of Nebraska-Lincoln are thanked for the support extended to this project.

### 6.7 References


Sanchis, J., Kantiani, L., Llorca, M., Rubio, F., Ginebreda, A., Fraile, J., Garrido, T. and Farre, M. 2012. Determination of glyphosate in groundwater samples using an ultrasensitive immunoassay and confirmation by on-line solid-phase extraction followed by liquid


CHAPTER 7. GENERAL CONCLUSIONS

Glyphosate is an herbicide that is sometimes used as pre-harvest desiccant or harvest-aid during wheat cultivation. The purpose of pre-harvest glyphosate application is enabling uniform harvest. Glyphosate’s herbicidal mode of action arises from its ability to inhibit a key enzyme, which is the 5-enolpyruvylshikimate-3-phosphate synthase enzyme in the shikimic acid pathway. As a result of this inhibition, the biosynthesis of aromatic amino acids is prevented. When using glyphosate as a harvest-aid, it is recommended to be applied at ripe stage of physiological maturity. In recent years, physiological and biochemical effects of glyphosate on plants have been investigated. Additionally, there is renewed interest regarding interactions between gut microbiota and glyphosate, as gut bacteria possess the shikimic acid pathway for the biosynthesis of aromatic amino acids. There have not been many studies that investigated the effects of pre-harvest glyphosate application on wheat quality and biochemical characteristics such as, protein and starch properties. Moreover, many gut microbiota related work has been performed in context of animals and not necessarily humans. In this context, this study was performed to address this knowledge gap. As such, the goals of this exploratory study were to determine the effects of pre-harvest application of glyphosate on wheat quality, protein and starch deposition in wheat as well as human gut microbiota. In order to reach these objectives, a field study and a greenhouse study were performed, where glyphosate was applied at soft dough stage and ripe stage. The field samples were analyzed for wheat quality characteristics, and other chemical properties such as starch, protein and shikimic acid levels. The greenhouse samples were analyzed for changes in the fine chemical properties of starch and proteins that occur from application until harvest. An in vitro fecal fermentation study was conducted with field study samples to analyze the interactions between glyphosate and human gut bacteria.
As for effects on wheat quality, the results indicated that the effects on quality are more pronounced when glyphosate is applied at soft dough stage as opposed to the ripe stage. In the analysis of the field study samples, the results showed that flour pasting properties, starch granules distribution and starch digestibility characteristics are impacted by glyphosate. Starch granule analysis demonstrated that pre-harvest glyphosate application lowers the proportion of B-type granules and vice versa for A-type granules. Significant differences were not observed for characteristics such as amylose and amylopectin ratio and molecular weight and starch pasting properties. The results of the greenhouse experiment indicated that starch amylopectin chain length was affected in different ways when glyphosate is applied at the different stages. However, the spatial distribution of starch granules and the morphology of granules were not affected. Glyphosate application also did not affect the primary and secondary structure of proteins as well as gluten protein composition, although glyphosate treated samples generally showed lower molecular weight values. Additionally, this study also showed that glyphosate causes the accumulation of shikimic acid, especially when applied at soft dough stage. As for the\textit{ in vitro} fecal fermentation study, the results showed that glyphosate may not have a profound effect on human gut microbiota, although the effects may not be consistent among individuals. Moreover, glyphosate appeared to have inhibitory effect on some species, such as \textit{Clostridium}.

As indicated earlier, previous work investigating the effect of glyphosate on different wheat related characteristics were not focused on determining the effects of this herbicide on wheat chemistry. The results obtained in this study are critical in answering the question ‘how does pre-harvest glyphosate timing affect wheat quality and the chemistry of starch and proteins?’ In this context, the results of the current study indicate that pre-harvest glyphosate application affects wheat quality characteristics such as, 1000-kernel weight, protein quality
characteristics and dough quality characteristics, especially when applied at soft dough stage. Additionally, shikimic acid accumulation was also induced by glyphosate, and the accumulation was greater when glyphosate was applied at soft dough stage. In previous studies, the accumulation of shikimic acid was shown to disrupt the carbon flow in affected plants, which may result in effects on other biosynthetic pathways. Thus, further studies should be conducted to understand the effects of shikimic acid accumulation with reference to wheat biochemistry.

This study also showed that pre-harvest glyphosate reduces the proportion of B-type granules, and that it affected wheat flour pasting properties. Average amylopectin chain length and the molecular weight of different fractions of gluten forming proteins was also reduced as a result of glyphosate. These observations could be caused by the interference of glyphosate in starch and protein deposition pathways. Such effects could also be consequences of shikimic acid accumulation and subsequent effects on carbon flow or the effects of glyphosate on enzyme activity caused by its metal chelation ability. Moreover, glyphosate may also affect intermolecular interactions between protein and starch, as well as other grain components such as, dietary fiber and lipids, which may be responsible for the observations made for dough and baking quality characteristics as well as flour pasting properties. Although it is unclear how changes in the chemical structure and composition of starch and proteins affect quality, there are indications that these changes may lead to variations in quality characteristics. With reference to the in vitro fecal fermentation study, the present study is the first that looked at glyphosate in the context of human gut microbiota. As previously stated, the results of this study show that although the effect of glyphosate on gut bacteria may not be consistent among individuals, glyphosate may cause inhibitory effects on some bacterial genera. This is an important finding as some bacterial genera are very important in maintaining overall gut health.
In conclusion, this study is the first of its kind, where the effect of pre-harvest application of glyphosate on wheat chemistry and human gut microbiota was investigated. Thus, the observations made in this study are crucial in elucidating the biochemical mechanisms and pathways affected by glyphosate, which warrant further research in this area.
CHAPTER 8. FUTURE WORK

This exploratory was insightful in gaining an in depth understanding about the many effects of glyphosate on wheat quality, wheat biochemical characteristics and human gut microbiota. As future work, further investigations can be performed to understand the biochemical mechanisms in which glyphosate affects properties such as, starch granules distribution, pasting properties, digestibility and protein molecular weight. By understanding such mechanisms, it would be possible to identify if and how glyphosate’s impact on the shikimic acid pathway causes changes in other plant biochemical processes. As some previous studies have indicated, shikimic acid accumulation could be one of the main reasons for glyphosate’s toxicity. Thus, finding the association between glyphosate’s mode of action, shikimic acid accumulation and other biochemical processes will be useful in gaining a complete understanding of the overall impact of pre-harvest glyphosate application on wheat chemistry.

The work on interactions between gut microbiota and glyphosate could be further expanded and can be carried out with many fecal donors with different dietary patterns and health conditions to get an overall picture as to whether glyphosate can affect the gut microbiome. Further *in vitro* experiments can be performed in environments with limited aromatic amino acid supply to determine if the inhibition of the shikimic acid pathway affects gut bacteria. Additionally, experiments can be designed to determine if glyphosate’s metal chelation capacity plays a role in its effect on the populations of gut bacteria.