Effect of genotype, growing environment, and fertilization treatment on free asparagine concentration in western Canadian wheat

by

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Preface

This thesis is written in manuscript style, including five chapters. Chapter 1 is the introduction for the study and Chapter 2 is the literature review, covering the basic knowledge of the elements studied throughout the thesis. Chapter 3 and 4 are research sections, which include two manuscript-style written chapters. A general discussion and conclusions for this study and recommendations for future research are presented in Chapter 5.

Contributions of Authors:

Chapter 3, "Effects of growing environment, genotype, and commercial fertilization levels on free asparagine concentration in Western Canadian wheat" was published in the focus issue- Grain and Grain-Based Food Safety in the journal Cereal Chemistry (Volume 98, Issue 1) authorship by Yi Xie, Lovemore Nkhata Malunga, Nancy P. Ames, John Waterer, Ali Salimi Khorshidi, and Martin G. Scanlon. Y.Xie was responsible for conducting the research and drafting the manuscript. L. N. Malunga and N. P. Ames were responsible for providing experimental methods and resources at Agriculture and Agri-Food Canada and giving critical advice on statistical analysis in the manuscript. J. Waterer was responsible for growing wheat samples in Paterson's research fields. A. S. Khorshidi was responsible for giving advice on statistical analysis and manuscript preparation and review. M. G. Scanlon was the corresponding author and was in charge of the research design and the reviewing of the manuscript. All authors agreed on the final version of the manuscript.

Chapter 4, "Comparison between a rapid analytical method and two well developed methods for wheat free asparagine detection and quantification" was prepared for journal publication with authorship by Yi Xie, Ali Salimi Khorshidi, Lovemore Nkhata Malunga, Rotimi E. Aluko, and Martin G. Scanlon. Y.Xie was responsible for conducting the research and drafting the manuscript. YA. S. Khorshidi was responsible for providing partial experimental resources, giving advice on experimental design, and manuscript preparation and review. L. N. Malunga were responsible for providing partial experimental methods and resources at Agriculture and Agri-Food Canada and giving advice on experimental design. R. E. Rotimi was responsible for manuscript review. M. G.

Scanlon was the corresponding author and was in charge of the research design and the reviewing of the manuscript. All authors agreed on the final version of the manuscript.

Abstract

The Maillard reaction, that involves a free amino acid – asparagine (ASN), as its main precursor, results in the formation of acrylamide in bakery products, such as bread. Acrylamide is known as a neurotoxin and a class 2A carcinogen to humans. Therefore, it is critical to understand how free ASN concentration in western Canadian wheat is influenced by wheat genotype, growing environment, and agronomic practices. Such an understanding allows strategies for controlling acrylamide in bread to be proposed. It is also important that quantification of free ASN for both research and industry occurs, and so a rapid test that allows accurate quantification of free ASN concentration becomes necessary.

Free ASN concentration in whole-wheat flour was measured using an ultrahigh performance liquid chromatography (UPLC) with a Photodiode Array Detector (PDA). The whole-wheat flour was milled from eight western Canadian wheat genotypes that were grown in three locations over two years, and that were subjected to four agronomic practices (two nitrogen levels combined with two sulfur levels). A rapid enzymatic test for asparagine concentration was validated against two established techniques using whole-wheat flour from a sub-sample set that consisted of all genotypes from all location-years with only one fertilizer treatment. For a preliminary study of bread acrylamide's relationship to wheat free ASN, six whole-wheat breads made from flour with low, medium, and high concentrations of free ASN were selected.

From the free ASN study, it was found that environment, genotype, and their interaction were the main influencing factors on free ASN concentration in wheat, whereas fertilization had a minimal effect (varied 521–557 $\mu g\ g^{-1}$ free ASN in wheat). The new rapid enzymatic test was reliable and user-friendly. A gratifying result was that the acrylamide concentration in the whole-wheat breads made with western Canadian wheat were all below 10 $ng\ g^{-1}$.

In conclusion, free ASN in Canadian wheat can be controlled with a careful selection of wheat genotype and nitrogen fertilization levels. In addition, the rapid enzymatic method is recommended for simple laboratory environments where free ASN analysis can be conducted at low cost.

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1 Introduction

Bakery products are one of the most popular foods around the world with a global consumption of 105.8 million tonnes in 2020, half of which (about 52.5 million tonnes) was consumed in countries of the European Union (Statista, 2021). Among the bakery products, bread accounted for a considerable segment (Statista, 2020), with over 2.3 million tonnes of bread sold in the UK alone in 2020 (Wunsch, 2021). Wheat is one of the earliest and most widely cultivated crops in human history (Atwell, 2001), and when ground to flour, it is the main ingredient of bread. Wheat is one of the most important crops in Canada and has been produced and exported the most compared to other grains (Canadian Grain Commission, 2021). In 2020-2021 alone, 28 million tonnes of wheat was produced, and 19.2 million tonnes were exported globally (Cereals Canada & Canadian Grain Commission, 2020). According to a report generated by the Canadian Grain Commission (CGC), in the period 2020 to 2021, over 59600 tonnes of Canadian wheat were exported to European countries, including Belgium, Italy, Portugal, Spain, and the United Kingdom, indicating that Europe is a crucial market for Canadian wheat (Canadian Grain Commission, 2021). From all the Canadian wheat produced last year, 21 million tonnes were from the Canadian Western Red Spring wheat class, a class that is known as the most suitable Canadian wheat for breadmaking (Cereals Canada & Canadian Grain Commission, 2020; Daker & Fréchette, 2001).

Even though there are enormous demands for Canadian wheat in the global market every year, attention should be paid to the grain safety issue associated with the acrylamide forming potential of wheat that has been explored in the past two decades (Stadler et al., 2002). Acrylamide as a "2a" carcinogen, which is formed through the Maillard Reaction during the bread baking process, was first identified in food in 2002 (Mottram, Wedzicha, & Dodson, 2002; Stadler et al., 2002). The Commission regulation of the European Union (EU) aims to ensure consumers are protected from potential adverse effects of the dietary intake of acrylamide (European Commission, 2017). However, given the fact that a consistent and mandatory action of controlling or reducing acrylamide levels in foods was absent, the European Commission required the European Food Safety Authority (EFSA) to monitor acrylamide levels in ten main categories of foods. In addition, EFSA was required to estimate the average daily dietary acrylamide exposure across all age groups (European Commission, 2017; European Food Safety Authority (EFSA), 2012). As a result, the EU established and reviewed acrylamide's benchmark levels in various foods, including in bread. The EU also requires the baking industries to devise strategies to reduce the acrylamide level in

bread (European Commission, 2017). The reviewed benchmark level of acrylamide in wheat bread by the EU commission regulation is 50 μg kg^{-1} (European Commission, 2017), whereas the acrylamide concentration in bread can range from 5 to 102 μg kg^{-1} (Abt et al., 2019). The Commission regulation regarding the acrylamide mitigation plan was made effective on April 11, 2018 (European Commission, 2017). Therefore, all wheat producers and bread manufacturers are required to demonstrate acrylamide reduction strategies so as to meet the requirements of this Commission regulation to ensure acrylamide levels in breads are lower than the benchmark.

Understanding the principles of the formation of acrylamide in bread is important for any acrylamide mitigation plan. It is known that acrylamide in bread is formed through the Maillard Reaction that involves amino and carboxyl compounds from the naturally present free amino acids and reducing sugars in wheat, which react at high temperatures (T > 120 ˚C), in processes such as baking, frying, and toasting (EFSA Contam Panel, 2015; JEFCA, 2005; Keramat, LeBail, Prost, & Jafari, 2011). In this reaction, free asparagine (ASN) has been reported to be the main precursor to acrylamide formation (Halford, Muttucumaru, Curtis, & Parry, 2007; Muttucumaru et al., 2008; Yaylayan, Wnorowski, & Perez Locas, 2003). During the breadmaking process, the Maillard reaction appears to be a surface phenomenon (Joint FAO/WHO Consultation, 2002) as about 99% of the acrylamide is concentrated in the crust of the bread (Capuano et al., 2009; Surdyk, Rosén, Andersson, & Åman, 2004). Thus, controlling the free ASN concentration in wheat is a potential solution for acrylamide mitigation in thermally treated wheat-based food products such as bread. Free ASN is present at its highest levels in the aleurone and bran layers of a wheat kernel (Shewry et al., 2009). As a result, whole-wheat flour has a higher acrylamide forming potential than white flour (Malunga et al., 2019; Shewry et al., 2009), i.e., whole-wheat bread contains more acrylamide than white bread (Ohm, Mergoum, & Simsek, 2017).

Investigating the factors that influence free ASN concentration is critical for reducing free ASN in wheat. Numerous research studies have reported that free ASN formation is mainly affected by three factors that include growing environment (Halford, Curtis, Chen, & Huang, 2015; Navrotskyi, Baenziger, Regassa, Guttieri, & Rose, 2018), wheat genotype (Liu, Ohm, Hareland, Wiersma, & Kaiser, 2011; Ohm et al., 2017), and agronomic practices such as nitrogen and sulfur fertilization treatments (Martinek et al., 2009; Weber, Koller, et al., 2008). For example, the free

ASN concentration in ground wheat wholemeal of seven varieties grown in Manitoba ranged from 379 to 965 μg g^{-1} (Malunga et al., 2019). Moreover, inadequate precipitation, drought, excessive heat due to high temperature and solar radiation, and stress due to disease are conditions that trigger free ASN accumulation in wheat grains (X. Gao, Lukow, & Grant, 2012; Giuliani, Giuzio, de Caro, & Flagella, 2011; Halford et al., 2015; Navrotskyi et al., 2018; Wilson, Guttieri, Nelson, Fritz, & Tilley, 2020). Furthermore, it has been reported that redundant nitrogen supplements and inadequate soil sulfur levels favor free ASN formation in wheat grains (Lea, Sodek, Parry, Shewry, & Halford, 2006; Stockmann et al., 2018). Thus, a good balance between nitrogen and sulfur fertilizer application is necessary for a free ASN reduction plan that is agronomically governed (Randall, Spencer, & Freney, 1981; Weber, Graeff, et al., 2008).

In order to implement approaches to comply with the regulations enforcing acrylamide reduction in food products, the concentration of free ASN needs to be determined in the raw ingredients, such as the wheat grain or its flours. Among various analytical techniques for free ASN measurement, two established techniques, UPLC and ^{1}H-NMR, have demonstrated reasonable robustness. These two analytical techniques have been adopted by various research groups around the world (Çelik & Gökmen, 2020; Corol et al., 2016; Malunga et al., 2021; Navrotskyi et al., 2018; Xie et al., 2021). But to have a rapid test method in a simple laboratory environment, or even at port for screening cargo shipments, is necessary to fulfill the emerging demand for free ASN analysis. A rapid enzymatic method coupled with Ultraviolet-Visible Spectrophtometer (UV-Vis Spec) that perfectly fits the needs for ASN analyses has been developed and applied to limited research studies in recent years (Lecart et al., 2018; Soares et al., 2020). However, a comprehensive comparison of the new method to the two established methods in terms of accuracy, precision, and efficiency in time and cost and ability to measure free ASN concentration in wheat grains is still lacking.

This thesis is a presentation of a comprehensive study of free ASN concentration in eight western Canadian wheat varieties grown under four environmental conditions subject to four combinations of nitrogen and sulfur fertilization treatments. In this way, a comprehensive assessment of genotype, environment, and agronomic practices on free asparagine content in Canadian wheat can be conducted. In addition, an extensive study to compare the quality parameters of three

analytical techniques and methods (^{1}H-NMR, enzymatic method, and UPLC) of free ASN detection in western Canadian wheat is conducted. The specific objectives pursued in this thesis are:

1. To study the effects of the growing environment (E), wheat genotype (G), and fertilization treatment (F) on free ASN concentration in western Canadian wheat grains.
2. To evaluate and compare the rapid enzymatic methods with the two established methods (UPLC and ^{1}H-NMR) for accuracy, precision, sensitivity, cost, and the ability to quantify free ASN concentration in whole-wheat flours.
3. To do a preliminary investigation of the relationship between free ASN in whole-wheat flour and acrylamide levels in the bread made from the flour.

The corresponding hypotheses of this thesis are:

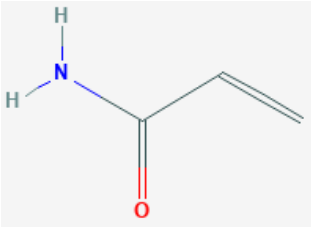
1. Environmental conditions play a significant role in free ASN formation in western Canadian wheat.
2. Three diverse detection methods for free ASN determination in whole-wheat flour are reliable.

2 Literature review

2.1 Acrylamide

Acrylamide (CH_2=CH-C(O)-NH_2) is an extremely hazardous, white, odourless, water-soluble, crystalline solid (Curtis & Halford, 2016a; Keramat et al., 2011; Lingnert et al., 2016; Raffan & Halford, 2019). It has a melting point of 84.5 ºC and a boiling point of 192.6 ºC at one atmospheric pressure (101.325 kPa) (Keramat et al., 2011; Lingnert et al., 2016; Riboldi, Vinhas, & Moreira, 2014) with a low molecular weight of 71.08 g mol^{-1}. The molecular structure is shown in Figure 2.1. Acrylamide is a biodegradable compound and has high mobility in soil and underground water due to its solubility in water (Lingnert et al., 2016; Raffan & Halford, 2019; Riboldi et al., 2014).

Figure 2.1: Acrylamide molecular structure. Figure from National Center for Biotechnology Information, Pubchem Compound Summary for CID 6579, acrylamide; [2021] – [cited 2021 Feb 22]. Available from https://pubchem.ncbi.nlm.nih.gov/compound/Acrylamide.



2.1.1 Acrylamide toxicity

In the last century, scientists devoted extensive efforts to study acrylamide, its properties, and toxicological effects. Acrylamide was then classified as a potential neurotoxin and group 2A carcinogen (probably carcinogenic to humans) by the International Agency for Research on Cancer in 1994 (International Agency for Research on Cancer, 1994). The harmful effects and toxicity of acrylamide were revealed from a water leakage accident in Sweden in 1997. Acrylamide has been used widely to produce polyacrylamide as a coagulant for water treatment and in the construction industry for erosion control (Kumar, Das, & Teoh, 2018; Lingnert et al., 2016; Riboldi et al., 2014). The accident happened during the construction of a tunnel and water leak to the surrounding environment, causing the death of fishes, cattle injury, and nerve system poisoning of several tunnel workers. It was found later that the leakage of acrylamide and N-methylolacrylamide from the tunnel wall caused this tragedy (Hagmar, Wirfält, Paulsson, & Törnqvist, 2005).

Since then, more researchers have studied the toxic effects of acrylamide in animals and human beings (Abramsson-Zetterberg, Wong, & Ilbäck, 2005; Matoso, Bargi-Souza, Ivanski, Romano, & Romano, 2019; T. Schettgen; B. Kutting; M. Hornig; M. W. Beckmann; T. Weiss; H. Drexler J. Angerer, 2004). In the in-vivo study carried out by Abramsson-Zetterberg et al. in 2005, the clinical signs of disease and neurotoxic, genotoxic, reproductive, and carcinogenic effects were shown in mice exposed to acrylamide (Abramsson-Zetterberg et al., 2005). Unfortunately, the adverse effects of acrylamide have also been detected on human health. The deterioration of the function of liver and nervous, immune, and reproductive systems are associated with acrylamide exposure (M. A. Friedman et al., 1986; Kumar et al., 2018; Matoso et al., 2019; T. Schettgen; B. Kutting; M. Hornig; M. W. Beckmann; T. Weiss; H. Drexler J. Angerer, 2004).

2.1.2. Dietary exposure to acrylamide

The presence of acrylamide (150-4000 μg kg^{-1}) in foods, mainly processed carbohydrate-rich foods, such as commercially processed potato, beetroot products and baked goods, was first reported by Tareke et al. in 2002. They also concluded that acrylamide concentration is very low (< 5 μg kg^{-1}) in unheated and boiled foods (Tareke, Rydberg, Karlsson, Eriksson, & Törnqvist, 2002). In the same year, two groups of researchers reported that the formation of acrylamide in heated food is due to the Maillard reaction that involved free asparagine (ASN) and reducing sugars (glucose, fructose, and maltose) at high processing temperatures (above 180 ºC) (Mottram et al., 2002; Stadler et al., 2002). Also, The Swedish National Food Administration and the University of Stockholm announced the presence of relatively high levels of acrylamide in high-temperature treated foods (Zhang, Zhang, & Zhang, 2005).

The discovery of acrylamide formation and its harmful effects drew great attention in the food industry and regulatory agencies around the world. In April 2003, the FAO and the WHO established an international network on acrylamide in food, which was used to share data relevant to the health risks of acrylamide in food (M. Friedman, 2003; World Health Organization, 2003). In Canada, Health Canada started to monitor the acrylamide content in food and to evaluate the efficacy of acrylamide reduction strategies in 2009. Food products from three classifications were monitored: foods commonly consumed by Canadians, foods considered important in terms of their

nutritional standpoint, and foods suspected of having high acrylamide concentrations (Health Canada, 2009). Later on, the Canadian Food Inspection Agency (CFIA) established a targeted survey (2009-2014) to collect data on a wide variety and range of potentially high acrylamide concentration commercial products, such as cookies, crackers, breakfast cereals, potato chips, and corn chips to monitor and assess the effectiveness of the implementation of acrylamide reducing strategies (Health Canada, 2017). In recent years, the European Commission established a database to summarize levels of acrylamide in common foods, issued a regulation on acrylamide mitigation measures, and recommended benchmarks for acrylamide levels in those common foods (Table 2.1) (European Commission, 2017).

Table 2.1: Distribution and benchmark levels of acrylamide in common food categories, using data from EFSA Panel on Contaminants in the Food Chain. (2015). Constructed using data from scientific opinion on acrylamide in food. *EFSA Journal*, *13*(6). https://doi.org/10.2903/j.efsa.2015.4104; and commission regulation (EU) 2017/2158. https://eur-lex.europa.eu/legal-content/en/txt/pdf/?uri=celex:32017r2158&from=en. Reprint permission from EFSA journal and journal of EU.

Food category	Mean ($\mu g\ kg^{-1}$)	P95 ($\mu g\ kg^{-1}$)	Benchmark ($\mu g\ kg^{-1}$)
French fries (ready to eat)	308	904	500
Potato crisps & snacks	389	932	750
Wheat soft bread	38	120	50
Other soft bread	57	240	100
Wheat and rye-based breakfast cereals	170	410	300
Crackers	231	590	400
Biscuits and wafers	201	810	350
Crisp bread	171	486	350
Roasted Coffee (dry)	249	543	400
Instant coffee (dry)	710	1122	850
Coffee substitutes (dry), based on cereals	510	N/A	N/A
Coffee substitutes (dry), based on chicory	2942	N/A	4000
Baby foods, other than cereal-based	24	72	N/A
Baby foods, processed cereal based foods for infants and young children excluding biscuits and rusks	26	83	40
Biscuits and rusks for infants and young children	111	287	150
Other products based on potatoes, cereals and cocoa	97	370	N/A

P95: 95^{th} percentile.

The direct dietary exposure to acrylamide among different age groups varies due to the variation of acrylamide levels present in different food categories (Table 2.2). Governments and international organizations around the world have been estimating, since 2002, the average dietary exposure of acrylamide for consumers and assessing the risk associated with acrylamide

containing foods (Abt et al., 2019; EFSA Contam Panel, 2015). In 2006, the Food and Agriculture Organization (FAO) and World Health Organization (WHO) Joint Expert Committee on Food Additives gathered data from 17 countries and reported that the average consumption of acrylamide for the general population is 0.3-2.0 μg kg^{-1} body weight per day, with the highest percentile of the population (99^{th} percentile) consuming over 5.0 μg kg^{-1} body weight per day (JECFA, 2006). Among all foods, a few categories contributed the most to the dietary acrylamide intake, including fried potato products (French fries (16 to 30%), potato crisps (6-46%), coffee (13-39%), pastry and cookies (10-20%), and breads (10-30%), while other food categories contributed less than 10% to the total dietary acrylamide intake (JECFA, 2006). In 2015, the European Food Safety Authority (EFSA) also measured the exposure to acrylamide among all age groups (Table 2.2). Both databases indicated that children consume about 2-3 times more acrylamide in their food than adults on a body weight basis because of two reasons: 1) children have significantly lower mean body weight compared to adults, and 2) the sources of food for children contain higher amount of acrylamide compared to those of other age groups (EFSA Contam Panel, 2015; JECFA, 2006).

Table 2.2: Acrylamide exposure level in all age general populations in μg kg^{-1} body weight per day. constructed using data from EFSA panel on contaminants in the Food Chain. (2015). Scientific Opinion on acrylamide in food. *EFSA Journal*, *13*(6). https://doi.org/10.2903/j.efsa.2015.4104. Reprint permission from EFSA journal.

Population age group	Mean	P95
Infants	0.8-1.0	1.8-2.1
Toddlers	1.3-14	2.3-2.4
Other children	1.2	2.2-2.3
Adolescents	0.7	1.4
Adults	0.5	1.0
Elderly	0.4-0.5	0.8-0.9
Very elderly	0.4-0.5	0.9

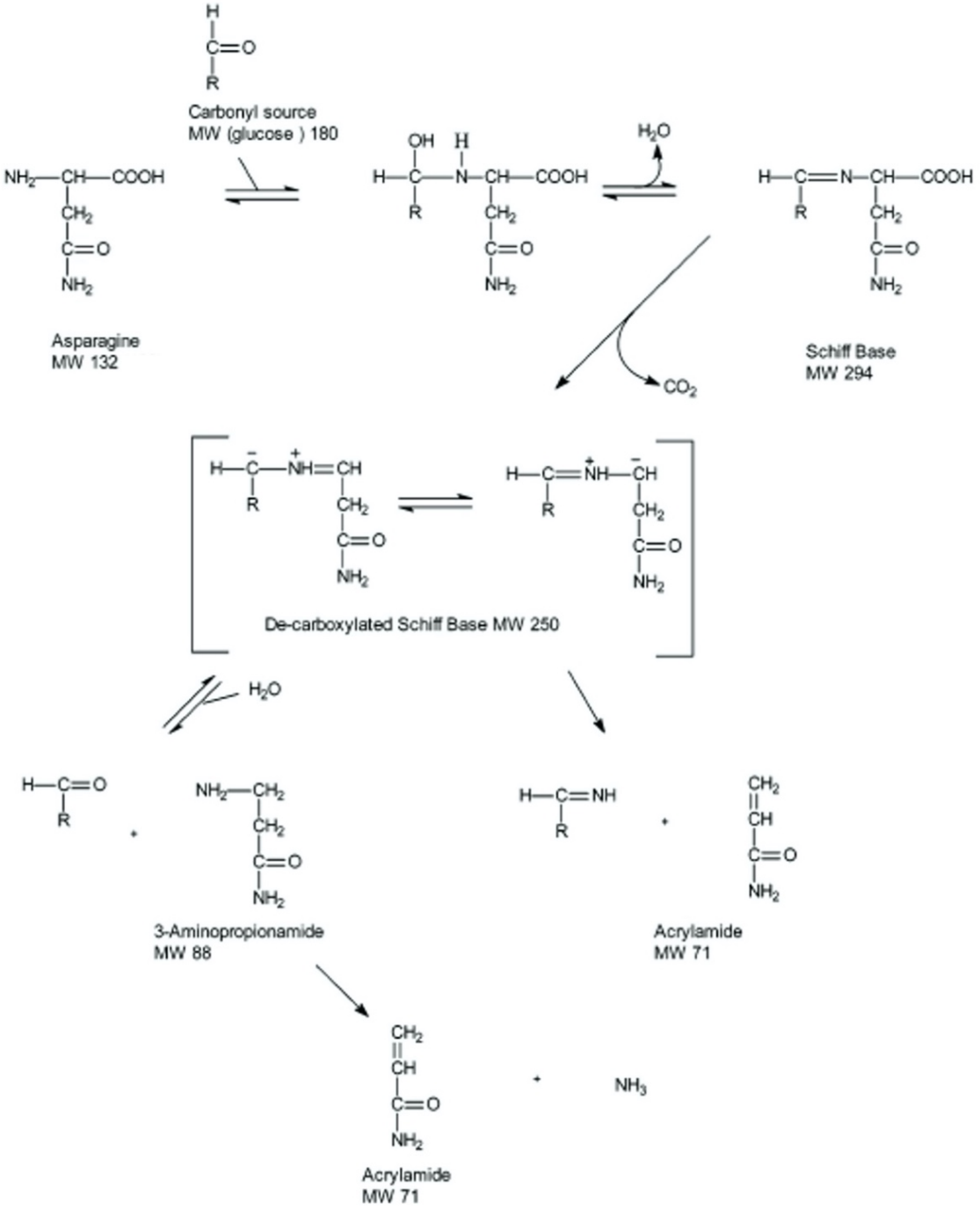
P95: 95th percentile.

2.1.3 Formation of acrylamide in food

Immediately after discovering the presence of acrylamide in common foods, researchers around the world started to study the precursors and mechanisms of the Maillard reaction for acrylamide formation. It was found that, in addition to the presence of free ASN and reducing sugars in food ingredients at high temperatures (above 120 ºC) (Mottram et al., 2002; Stadler et al., 2002), treatment with low moisture content food ingredients is also a critical factor leading to acrylamide formation (EFSA Contam Panel, 2015; Joint FAO/WHO Consultation, 2002; Keramat et al., 2011).

During the thermal processing of food ingredients containing free amino acids and reducing sugars, the α-amino group of free ASN reacts with the carbonyl group of the reducing sugar to form an intermediate product - a Schiff base (Figure 2.2) - by losing one water molecule (M. Friedman, 2003; Keramat et al., 2011; Lingnert et al., 2016; Raffan & Halford, 2019; Vattem & Shetty, 2003; Yaylayan et al., 2003). Under high temperature treatments, the Schiff base undergoes a decarboxylation that involves the delocalization of a negative charge to form a decarboxylated Schiff base. There are two potential pathways to form acrylamide after this stage: 1) the decarboxylated Schiff base loses an imine to form acrylamide directly, or 2) the decarboxylated Schiff base undergoes a hydrolysis process to form 3-aminopropionamide, which is then decomposed into acrylamide (Figure 2.2). In this reaction, free ASN provides the C-backbone for the Schiff base and the acrylamide (Weisshaar, 2004; Zyzak et al., 2003).

Figure 2.2: Mechanism of acrylamide formation from free asparagine and reducing sugar. Reprinted (adapted) with permission from Zyzak et al. (2003). Copyright © 2003, American Chemical Society.



2.1.4 Acrylamide in wheat bread

Acrylamide is present in various baked cereal-based products (Table 2.1) such as bread, which is one of the most popular cereal-based products consumed daily by people around the world (Capuano & Fogliano, 2011). Baked products contribute about 25% of the total acrylamide dietary intake for consumers, with bread being the main contributor that accounted for about 11% of the total intake, i.e., about 31 μg day^{-1} (Claus et al., 2006; Granby et al., 2008; Keramat et al., 2011). Therefore, controlling the concentrations of acrylamide in bread could help to reduce the daily intake of acrylamide for consumers. Acrylamide levels in bread may be influenced by several factors, including wheat flour bread formulation, fermentation conditions, and baking conditions. After bread has been baked, the acrylamide levels in the bread may be reflected by the color of the bread crust (Claus et al., 2006; Mustafa, Andersson, Rosén, Kamal-Eldin, & Åman, 2005).

Wheat flour, as the most important ingredient of bread, plays a critical role in acrylamide formation during breadmaking. The variation of extraction rate of wheat flour during the milling process would lead to different concentrations of ash, free ASN and reducing sugars, as well as differences in protease and amylase activities. All these factors could influence the acrylamide levels in bread (Capuano, Garofalo, Napolitano, Zielinski, & Fogliano, 2010; Claus et al., 2006; Fredriksson, Tallving, Rosén, & Åman, 2004; Malunga et al., 2019). As shown in a study conducted by Fredriksson et al. (2004), increasing the extraction rate of wheat flour from sifted flour (73- 78%) to whole grain flour (100%) resulted in over a 200% increase in free ASN concentration (Fredriksson et al., 2004). In another study carried out by Claus et al. (2006), when the extraction rate during milling of wheat flour increased from 80 to 100%, the free ASN concentrations in wheat flours increased by 3.5 fold (1.37 to 4.85 mg kg^{-1}) (Claus et al., 2006). Meanwhile, protease and amylase activities increased with an increase in flour extraction rate, leading to higher concentrations of ASN and reducing sugars in the resulting flour (Claus et al., 2006) . The increasing wheat flour extraction rate resulted in the acrylamide concentration in bread increasing by over two fold (from 45.5 to 115.3 μg kg^{-1}). Shewry et al. (2009) hand-dissected wheat grains into fractions, and reported that free ASN is mainly concentrated in the aleurone layer (about 246 mg kg^{-1}) and wheat bran (about 589 mg kg^{-1}) (Shewry et al., 2009). Their findings explained how extraction rate affected the acrylamide concentration in bread, as higher proportions of free ASN

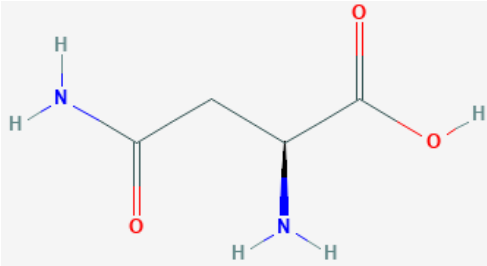
and reducing sugars in flour milled at higher extraction rates will eventually lead to higher concentrations of acrylamide in the baked product.

Compared to free ASN concentration, the concentration of sugar is very high in a wheat plant (Ciesarová, 2016; Corol et al., 2016; Hamlet, Sadd, & Liang, 2008). That is, the free ASN concentration in the whole grain flour is about 374 mg kg^{-1}, whereas the presence of reducing sugar is about 8460 mg kg^{-1} (Hamlet et al., 2008). Thus, free ASN is the limiting factor for the formation of acrylamide through the Maillard reaction. This makes free ASN the main precursor of acrylamide, which means that free ASN governs the amount of acrylamide formed in the bread (Curtis et al., 2009; Granby et al., 2008; Muttucumaru et al., 2008).

2.2 Free Asparagine in Wheat

Free ASN (H_2N-CO-CH_2-CH(NH_2)-COOH) is one of the non-essential amino acids to humans, with a molecular weight of 132.12 g mol^{-1} and an isoelectric point of 5.41, containing an amino group and a carboxylic group indicated in Figure 2.3 (Ciesarová, 2016; Lea et al., 2006). ASN can be dissolved in both acid and alkali, but it has a moderate solubility and tends to form white crystals in water (Lea et al., 2006). In plants, some of the nitrogen (N) atoms are present in protein, but some are not, with the latter being called non-protein N (Lea et al., 2006). Free ASN, which contains non-protein N atoms, exists in a wide range of plants such as potato, cereal grains (wheat, rye, oat, maize), coffee beans, vegetables (asparagus, onion, broccoli, pumpkin), fruits (plum), seeds (peanut, soybean), and others (chicory) (Ciesarová, 2016). More specifically, in potato, free ASN is the primary accumulation site for non-protein N contributing to about 33-59% of the total free amino acids pool (Lea et al., 2006). Similarly, free ASN is one of the most dominant free amino acids in wheat, which makes up to 30% of the total free amino acid pool (Curtis et al., 2009; Muttucumaru et al., 2008). Therefore, understanding the factors that influence the free ASN formation in wheat becomes critical in controlling its concentration.

Figure 2.3: Asparagine molecular structure. Figure from National Center for Biotechnology Information, Pubchem Compound Summary for CID 6267, asparagine; [2021] – [cited 2021 Feb 22]. available from https://pubchem.ncbi.nlm.nih.gov/compound/Asparagine.



2.2.1 Factors affecting free ASN in wheat

A tremendous amount of research has been conducted on possible solutions to reduce the free ASN concentrations in wheat (Halford et al., 2015; Malunga et al., 2021; Ohm et al., 2017; Stockmann et al., 2018; Yadav, Carroll, Estavillo, Rebetzke, & Pogson, 2019). Scientists discovered that free ASN concentration in wheat is mainly influenced by genotype, environmental conditions (drought, heat and soil characteristics) and agronomic practices (Curtis & Halford, 2016a; Curtis, Powers, Wang, & Halford, 2018; Navrotskyi et al., 2018; Ohm et al., 2017; Raffan, Oddy, & Halford, 2020; Weber, Koller, et al., 2008).

2.2.1.1 Genotype (G)

Wheat genotype has been demonstrated to have a significant influence on wheat free ASN (Curtis et al., 2009; Malunga et al., 2021; Navrotskyi et al., 2018; Ohm et al., 2017). Corol et al. (2016) reported the free ASN concentration range in 26 varieties of wheat grown under six environments in four countires (the UK, France, Poland, and Hungary) to be 408-791 µg g^{-1}. In these selected 26 wheats, the effect of genotype explained 13% of the total variation of free ASN concentration, which was significant (Corol et al., 2016). As per Ohm et al. (2017), genotype contributed up to 27% of the total variance in free ASN concentration in 75 Hard Red Spring wheat varieties grown at three locations in North Dakota. The authors reported a free ASN concentration range of 357 to 1037 µg g^{-1} (Ohm et al., 2017). Malunga et al. (2021) studied 8 Canadian wheat varieties grown at two locations, and reported a free ASN concentration range of 322 to 891 µg g^{-1}. Genotype was

responsible for 14% of the total variation in free ASN concentration of these Canadian wheat varieties (Malunga et al., 2021).

Variation in free ASN concentrations among wheat varieties is influenced by the ASN synthetase gene family, which consists of four genes (TaASN1, TaASN2, TaASN3, and TaASN4) (Curtis, Bo, Tucker, & Halford, 2018a; Curtis & Halford, 2016b; R. Gao et al., 2016; Raffan & Halford, 2019). These four synthase genes play an important role in catalyzing the transfer of an amino group from glutamine to aspartate, thus facilitating the formation of ASN and glutamate (Curtis, Bo, et al., 2018a; R. Gao et al., 2016; Raffan & Halford, 2019). Among these four genes, nutrient availability has been shown to impact TaASN1 and TaASN3 (R. Gao et al., 2016). Between the two genes, TaASN1 has been shown to be most responsive to the availability of N and deficiency of S (Curtis et al., 2019; R. Gao et al., 2016; Raffan & Halford, 2019). TaASN2 is the main asparagine synthetaze gene that is expressed during the mid to the late grain development period in any tissue, with its highest expression levels ocurring in the embryo and endosperm of wheat grains (R. Gao et al., 2016; Raffan & Halford, 2019). Being present in different organs of wheat, TaASN3 expression is responsive to varying N and S levels (R. Gao et al., 2016; Raffan & Halford, 2019). Expression level of TaASN4 is low compared to that of other three genes (Raffan & Halford, 2019). To the best of our knowledge, the number of studies on TaASN4 is still very limited as it has only been discovered recently (Curtis & Halford, 2016b; Curtis et al., 2019; Xu et al., 2018).

2.2.1.2 Environment (E)

Growing environment contributes significantly to free ASN formation in wheat (Curtis & Halford, 2016b; Martinek et al., 2009; Parisi et al., 2018; Yadav et al., 2019). The growing conditions associated with drought, soil water retention, precipitation, heat and solar radiation, as well as stress due to disease were shown to largely impact free ASN concentrations in wheat (Curtis, Bo, Tucker, & Halford, 2018b; Lea et al., 2006; Navrotskyi et al., 2018; Wilson et al., 2020).

Drought and heat stress are two critical environmental constraints that remarkably affect plant growth, crop yield, and reproduction (Good & Zaplachinski, 1994; Kusaka, Ohta, & Fujimura, 2005; Sattar et al., 2020). Drought stress could be related to limited availability of water in the soil

and low precipitation (Sattar et al., 2020), and these trigger formation of high levels of free ASN in wheat, especially during the grain filling period (Corol et al., 2016; Graybosch, Peterson, Baenziger, & Shelton, 1995; Lea et al., 2006; Navrotskyi et al., 2018; Yadav et al., 2019). As per Kusaka et al. (2005), the concentration of free amino acids started to increase in pearl millet after irrigation ceased, with free ASN and proline being the two amino acids that accumulated the most. Concentration of free ASN in crop stem and leave increased by over 10 and 23 fold, respectively, during nine days without irrigation (Kusaka et al., 2005). The accumulation of free ASN was seen as a good sign of protein and amino-acid degradation as a response to drought-stress or other stress conditions (Brouquisse, James, Pradet, & Raymond, 1992; Curtis, Bo, et al., 2018b; Good & Zaplachinski, 1994; Sotero-Martins, Da Silva Bon, & Carvajal, 2003).

Good & Zaplachinski (1994) explored similar trends in *Brassica napus* plants where free ASN concentration in the plant increased by 5 and 13 fold after two and four days of drought, respectively. In the meantime, protein synthesis was negatively affected by drought stress (Good & Zaplachinski, 1994). Therefore, an increase in free amino acids is a responsive process to drought that occurs in a plant (Good & Zaplachinski, 1994). Yadav et al. (2019) studied the effect of drought conditions on free ASN accumulation in wheat plants and they observed an increase in free ASN and a decrease in grain yield due to drought (Yadav et al., 2019). In the meantime, the amount of free ASN that accumulated in the wheat plant is negatively associated with wheat drought tolerance, which means that the amount of free ASN tends to increase in the wheat plant that has low tolerance to drought stress. Therefore, the accumulation of free ASN is an indicator of the drought tolerance (Yadav et al., 2019).

Apart from drought caused by low precipitation, soil with low water retention capacity will also cause high levels of free ASN formation (Martinek et al., 2009). Soil water retention is one of the characteristics associated with soil type. For example, clay type soil has greater water retaining capability compared to the sandy-like black and Gray Chernozem soils (Fuller, 2010; Kandel et al., 2013). More specifically, Chernozem soil is a sandy-like soil that has larger particle size (average of 0.075 to 4.75 mm particle size) compared to a clay type soil (average of 0.002 to 0.075 mm particle size) (Briaud, 2013). The difference in particle size made the Chernozem type soil a non-waterlogged soil, meaning that water tends to drain away from the soil (Fuller, 2010).

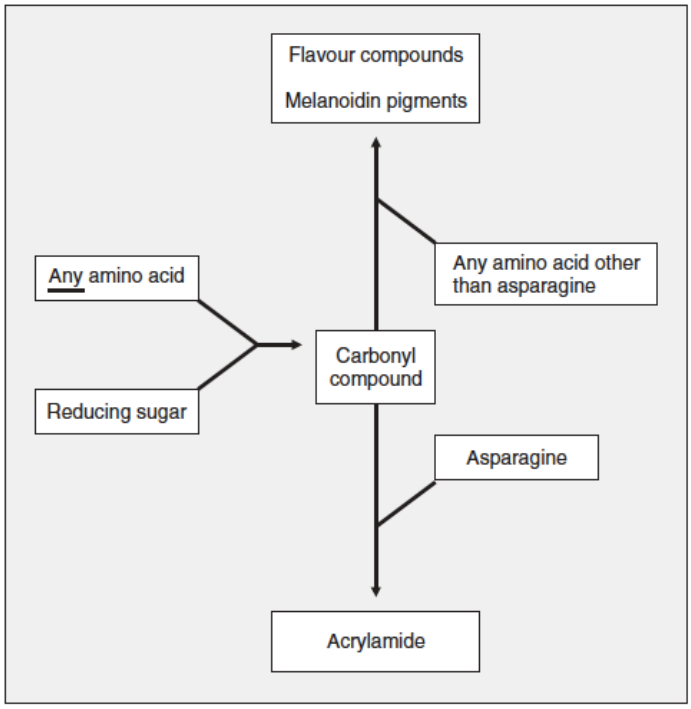
Therefore, even under the same precipitation or irrigation conditions, Chernozem soil will hold less water, and so will be more prone to drought compared to clay soils.

Stress due to heat is also a factor that triggers free ASN formation in wheat (Corol et al., 2016; Lea et al., 2006; Navrotskyi et al., 2018). As per Navrotskyi et al. (2018), heat stress induced by solar radiation resulted in longer wheat growing periods from flowering to harvest, leading to higher free ASN accumulation in wheat.

2.2.1.3 Fertilization (F)

One clear trend associated with the application of N fertilizer is an increase in total free amino acids in wheat crops, promoting the formation of acrylamide in wheat-based products processed at high temperatures (Halford et al., 2007). As per Mottram et al. (2002) & Stadler et al. (2002), increasing total amino acid content in wheat flour led to an increase in carbonyl intermediate substances formed during the Maillard reaction. With the presence of free ASN, the carbonyl intermediate substance will further transform into acrylamide, whereas other free amino acids will react with the rest of carbonyl intermediate substances and form flavouring compounds (Figure 2.4) (Halford et al., 2007).

Figure 2.4: Free amino acids reacting with the carbonyl compound during the Maillard reaction (Halford et al., 2007). Figure 5 from N. G. Halford, N. Muttucumaru, T. Y. Curtis & M. A. J. Parry (2007) Genetic and agronomic approaches to decreasing acrylamide precursors in crop plants, Food Additives & Contaminants, 24:sup1, 26-36, DOI: 10.1080/02652030701403093. Reprinted of permission from Taylor & Francis Journal.



As reported by Claus et al. (2006), N fertilizer had a significant impact on free ASN and crude protein concentration in wheat grown in Germany. Free ASN increased by almost 300%, and crude protein increased by almost 100% after applying 200 kg ha^{-1} N fertilizer. As a result of an increase in N fertilizer, acrylamide levels in bread increased by about 300% (Claus et al., 2006). Martinek et al. (2009) observed a similar trend from a study carried out in the Czech Republic, i.e., a gradual increase in N fertilizer from 0 to 180 kg ha^{-1} (by applying 60 kg ha^{-1} N fertilizer in three steps throughout the growing season) resulted in about a 150% increase in free ASN concentration in wheat in two growing years (Martinek et al., 2009). To minimize the acrylamide forming potential, a careful selection of the right amount of N fertilizer to support wheat plant growth, amino acids, and protein formation is critical. Therefore, farmers could control free ASN by agronomic practices while saving the cost of N fertilization (Claus et al., 2006; Halford et al., 2007). However, the role of fertilization in free ASN formation can not be fully understood by focusing on the

implementation of N fertilizer alone, as S fertilizer also played a critical role in free ASN formation in wheat (Curtis, Powers, et al., 2018; Halford et al., 2007; Li, Tyl, Kaiser, & Annor, 2019; Liu et al., 2011; Shewry et al., 2009; Stockmann et al., 2018; Wilson et al., 2020).

Sulfur concentration in the soil has been shown to have a significant influence on the amino acid profile and protein composition in wheat (Halford et al., 2007; Shewry et al., 2009). When S is adequately supplied to soil, wheat plant tends to yield more S-rich amino acids, such as methionine and cysteine, and more S-rich storage proteins, including α- and γ-gliadin and low molecular weight glutenins in the grain (Curtis et al., 2009; Granvogl, Wieser, Koehler, Von Tucher, & Schieberle, 2007; Raffan et al., 2020; Zhao, Hawkesford, & McGrath, 1999). Sulfur deficient soils yield grains with more S-poor amino acids, such as ASN, alanine, and glutamine, as well as S-poor storage proteins, mainly ω-gliadin and high molecular weight subunits of glutenin (Li et al., 2019; Raffan et al., 2020; Shewry, Franklin, Parmar, Smith, & Miflin, 1983; Steinfurth, Mühling, & Zörb, 2011; Zhao et al., 1999).

The effect of S on free ASN concentration is strongly associated with the soil N to S ratio (Randall et al., 1981; Stockmann et al., 2018). Grain S content is strongly associated with application of S into the soil (Muttucumaru et al., 2006a). Supplying S to wheat grown under S deficiency conditions led to a huge change in the free amino acid pool and a significant increase in free ASN accumulation compared to supplying S to the wheat grown with sufficient S availability (Curtis et al., 2009). Grains would be considered S deficient if S content is less than 0.12% or the N to S ratio is larger than 17 in the grain (Granvogl et al., 2007; Stockmann et al., 2018). This means that applying S fertilizers would be effective in reducing wheat free ASN in S deficient conditions. However, application of S would not change free ASN formation when soil has sufficient S (Stockmann et al., 2018; Weber, Koller, et al., 2008). The Home Grown Cereals Authority reported that about 23% of the UK suffered from S deficiency (Halford et al., 2007). Moreover, some areas in Northern and Western Europe, Australia, and New Zealand have soil S deficiency concerns (Halford et al., 2007; Muttucumaru et al., 2006a). Therefore, the application of S to the soil in these areas is extremely effective in reducing free ASN in their crops (Curtis et al., 2009; Elmore, Parker, Halford, Muttucumaru, & Mottram, 2008; Muttucumaru et al., 2006b). For example, free ASN concentration in wheat decreased over 20 fold by applying 40 kg S ha^{-1} to the Rothamsted

Research Farm in the UK (Muttucumaru et al., 2006b). However, a completely different trend was observed by researchers in Germany, i.e., when the wheat plant had adequate S during the grain filling period, application of S had no impact on the concentration of free ASN in wheat grains (Stockmann et al., 2018; Weber, Koller, et al., 2008). Therefore, a judicious selection of agronomic practices by considering the effect of N and S fertilizers and soil conditions is necessary for controlling free ASN concentration in wheat.

Due to differences in growing environments, wheat varieties and fertilization treatments used in different studies, the extents to which these factors affected wheat free ASN vary (Corol et al., 2016; Ohm et al., 2017). For example, a study by Ohm et al. (2017) indicated that the growing location contributed the most to free ASN formation in wheat (about 32.5%) among factors such as genotype, growing environment and the interaction between them, whereas genotype and the interaction between growing environment and genotype accounted for 26.6% and 4.6% of the variation in free ASN concentration, respectively. As per Corol et al. (2016), who compared the effects of genotype, growing environment and their interactions on wheat free ASN, its concentration was influenced mostly by the interaction between genotype and growing environment (44%), followed by environment (35%), and genotype (13%). In addition, free ASN concentration in wheat was found to respond differently to fertilization treatments under different environmental conditions (Curtis et al., 2009; Weber, Koller, et al., 2008). Free ASN levels in wheat were reduced drastically after S application in a study carried out in the UK on S deficient soils (Curtis et al., 2009; Raffan & Halford, 2019), while the free ASN levels in wheat were not significantly changed by S fertilization when non-S-deficient soils were used (Stockmann et al., 2018; Weber, Koller, et al., 2008).

2.2.2 Free ASN analysis techniques

There are various analytical methods used for free ASN analysis by different research groups, such as ^{1}H-nuclear magnetic resonance (NMR) (Corol et al., 2016; Navrotskyi et al., 2018), enzymatic methods coupled with ultraviolet-visible spectrophotometry (UV-Vis spec) (Lecart et al., 2018; Soares et al., 2020), and liquid chromatography (LC) with different detection techniques (Granby et al., 2008; Xie et al., 2021). Even though there are different drawbacks and benefits of selecting one analytical method over the others (Claridge, 2016; Megazyme, 2018b; Waters Corporation,

2016), to the best of our knowledge, the rationale and reasons for the selection of a specific analytical method for free ASN analysis is not specified in most literature. It is nevertheless crucial to understand the advantages and disadvantages associated with a given method. As a result, depending on the circumstances, one can select a suitable analytical method that can meet the needs of analysis of free ASN, in terms of instrument availability, the robustness and quality of analysis (including data accuracy, precision, sensitivity, and limit of quantification (LOQ)), and the efficiency of analysis from cost and time perspectives. A review of three main techniques for free ASN analysis is therefore conducted.

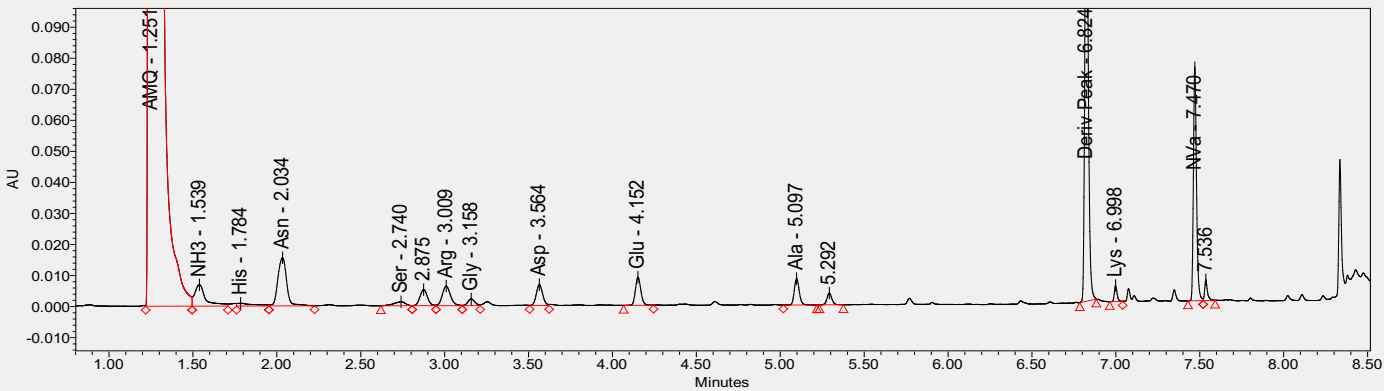
2.2.2.1 UPLC-PDA method

Amino acid analysis and quantification has always been an integral part in the life sciences, natural sciences, and food science (Alterman, 2019; Salazar, Armenta, Corte´s, & Shulaev, 2019). There are multiple methods and protocols of amino acid analysis using chromatography in modern analytical chemistry (Alterman, 2019). In the past decade or so, the techniques of amino acids analysis experienced drastic change with more application of the use of mass spectroscopy (MS) as a new detection method. Also, the improvement of old amino acid analysis techniques has occurred, for example, the introduction of AccQ-Tag derivatization method coupled with ultra-performance liquid chromatography (UPLC) has now gradually replaced the use of the old version of HPLC with a fluorescence detection method (Alterman, 2019). More research groups have adopted UPLC as their amino acid analysis tool (Boughton et al., 2011; Çelik & Gökmen, 2020; Graziano et al., 2019). Free amino acid analysis is one important sub-category of amino acid analysis (Salazar et al., 2019). More specifically, the use of the well-developed, robust, and efficient UPLC technique is commonly applied for free amino acids detection and quantification more recently (Malunga et al., 2019; Qiu, Reynolds, Johanningsmeier, & Truong, 2020; Xie et al., 2021). The combination of UPLC with AccQ-Tag derivatization has become one of a popular method for free ASN analysis in food materials (Salazar et al., 2019). AccQ-tag derivatization is one pre-column derivatization method, which involves the use of 6-aminoquinoly-N-hydroxysuccinimidyl carbamate (AccQ) derivatives (Salazar et al., 2019). The purpose of using AccQ derivatives is to react with the amine group in free amino acids to form stable fluorescent compounds, so that the derivatized free amino acids can be detected using a Photodiode Array detector (Cohen, 2005; Weiss, 2005). This pre-column derivatization method has very high

sensitivity that only requires a very small sample volume for free ASN analysis (Salazar et al., 2019). Reversed-phase-UPLC separation after a pre-column derivatization for free amino acid analysis has gained acceptance due to better efficiency and sensitivity, and shorter sample elution times (Salazar et al., 2019). Reversed-phase-UPLC separates free amino acids based on the polarity. It allows the polar free amino acids to elute faster than the non-polar free amino acids (Qiu et al., 2020). The total elution time (or retention time) for each free amino acid analysis through UPLC is 10.2 minute, with the elution time of free ASN being approximately 2 minutes (Figure 2.6). In this analysis, 250 μM L-Norvaline (NVA) is incorporated as an internal standard into the free amino acid extraction solution for free ASN quantification purposes. Similar to the ^{1}H-NMR method, the advantage of using an internal standard is to improve the accuracy of free ASN concentration quantification. In each UPLC chromatogram, the concentration of Free ASN is quantified based on the ratio between integration of the free ASN peak to the integration of the L-NVA peak for each sample, thereby eliminating variability in the free ASN and L-NVA recovery rate during the extraction process among each sample (Xie et al., 2021). The free ASN concentration is quantified based on the ratio of integration of the ASN peak relative to the integration of NVA peak.

Various free ASN analyses have been conducted to study the quality parameters of the UPLC-PDA method (Malunga et al., 2019; Waters Corporation, 2007, 2016). In terms of the accuracy levels of the UPLC technique, as per Waters UPLC Amino Acid Analysis Solution System Guide (2007), high accuracy at pico-molar concentration levels is an important advantage of using the AccQ-Tag method for free ASN analysis (Waters Corporation, 2007). For the precision of the technique, as reported by Malunga et al. (2019), the coefficient of variation (CV%) of the ASN analysis using UPLC ranged from 0.3 to 10.0% (Malunga et al., 2019). Lastly, great sensitivity and relatively low LOQ are advantages associated with UPLC technique. As per Waters UPLC Amino Acid Analysis Solution - System Guide (2007), UPLC is able to accurately quantify samples with free ASN concentrations above 6.606×10^{-3} mg L^{-1} (Waters Corporation, 2007).

Figure 2.5: An example of a typical chromatograph for free amino acid analysis using UPLC with a PDA detector. The chromatograph is generated by using whole-wheat flour sample made with the genotype BW5011 that was grown in 2018 at Lilyfield under 100 kg nitrogen ha^{-1} fertilization treatment as reported in Xie et al. (2021).

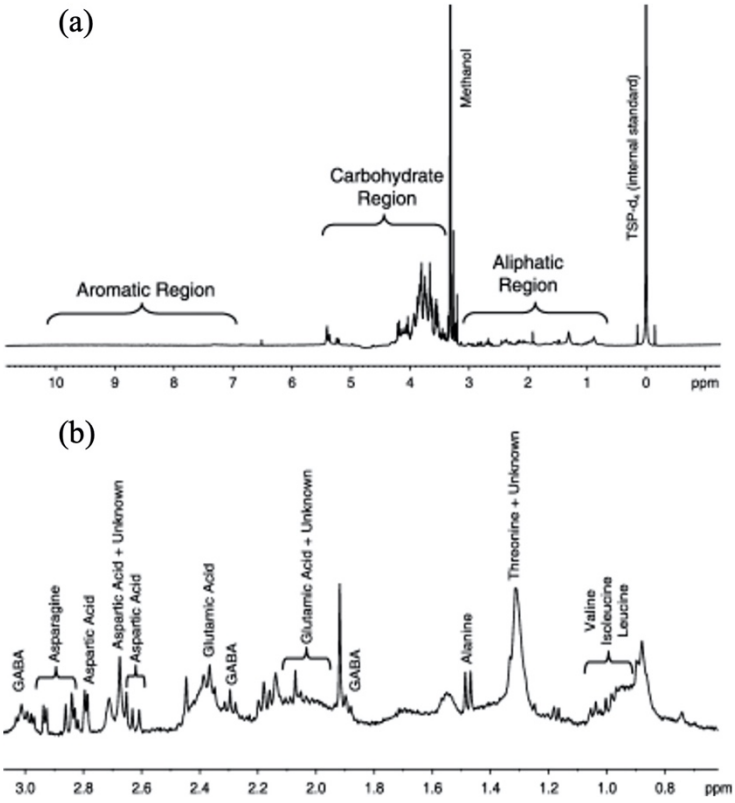


2.2.2.2 ^{1}H-NMR method

^{1}H-NMR is a relatively novel technique in food science research that had limited applications until the end of the last century (Hatzakis, 2019). In analytical chemistry, ^{1}H-NMR is one of the most powerful and robust technique available for structural analysis of unknown molecules (Ward & Beale, 2006). Compared to the ^{1}H-NMR technique, chromatography techniques, with mass spectroscopy or photodiode array detector, require more effort in the sample preparation stage. Very often, a derivatization step is required to attach a fluorescent compound to the compound of interest (Cohen, 2005) because chromatography can only detect and analyze a compound that is volatile, and this is achieved by the derivatization procedure (Ward, Harris, Lewis, & Beale, 2003). ^{1}H-NMR is a non-destructive technique that does not require purification steps to segregate certain types of chemical compounds during sample preparation (Hatzakis, 2019; Ward & Beale, 2006). For example, in the ^{1}H-NMR analysis of primary plant metabolites for mulberry leaves conducted by Liang et al (2018), ^{1}H-NMR was able to detect and analyze three types of chemical compounds (amino acids, carbohydrate, and alkaloids) in the spectrum. In food science, ^{1}H-NMR is used to analyze the structure of complex food compounds and to understand molecular interactions and the food matrix for a wide range of food materials, such as beverages, oils and lipids, vegetables, meats, and dairy, principally in the most recent two decades (Defernez et al., 2004; Hatzakis, 2019). More specifically, the ^{1}H-NMR technique started to be utilized extensively for plant metabolites and analysis of organic compounds, such as amino acids, carbohydrates, lipids and

fatty acids (Corol et al., 2016; Ward & Beale, 2006). In recent years, the ^{1}H-NMR technique was utilized to measure free ASN concentration in whole-wheat flour samples. In this analysis, deuterated NMR solvent that contains 80:20 deuterium oxide: deuterated methanol (D_2O: CD_3OD) with 0.05% w v^{-1} TSP-d4 (sodium salt of trimethylsilyl propionic acid) is added to the whole-wheat flour sample in order to extract free ASN from the supernatant. Direct extraction of a whole-wheat flour sample that contains the target analyte (free ASN) into the NMR solvent is a great advantage of this free ASN extraction method. Additional processes for sample purification, such as evaporation and re-dissolution of the extracted free ASN is not required. This lowers the risk of losing the target analyte (free ASN) during sample purification (Ward et al., 2003). Free ASN concentration was quantified based on the number of hydrogen atoms present in free ASN molecules in the whole-wheat flour. During the quantification process, TSP-d4 with a constant concentration was added as an internal standard to each sample (Baker et al., 2006; Corol et al., 2016; Ward et al., 2003). As in figure 2.5, the signal of TSP-d4 appeared at the 0.0 ppm (parts per million) region of the spectrum and was integrated as 1 (Hatzakis, 2019). The signal region for free ASN is between 2.86 and 2.96 ppm from the spectrum (Liang et al., 2018). The concentration of free ASN is quantified based on the ratio of the integration of the peak for TSP-d4 relative to the integration of the free ASN peak. For example, if the ratio of TSP-d4 peak to the free ASN peak is 1 to 0.005 in a whole-wheat flour sample, then the concentration of TSP-d4 is 200 fold that of the concentration of free ASN in the sample (Baker et al., 2006; Liang et al., 2018; Ward et al., 2003).

Figure 2.6: Example of a typical ^{1}H-NMR spectrum of wheat flour extract. (a) full ^{1}H-NMR spectrum from 0 to 10 ppm (tsp-d4 as an internal standard with chemical shift at 0.0 ppm); (b) enlarged spectrum from 0.8 to 3.0 ppm (free asn with chemical shift at about 2.8 to 2.96 ppm). source: john m. baker et al. (2006), figure 1, p. 384. reproduced with permission of john wiley and sons.



In terms of quality parameters for the ^{1}H-NMR method, researchers discussed the accuracy, precision, sensitivity, and LOQ in different works. As per Liang et al. (2018), the accuracy of the ^{1}H-NMR method for free ASN quantification was about 118.5%, which means the measurements usually are greater than the true value (Liang et al., 2018). For the precision of the technique, as reported by Corol et al. (2016), the coefficient of variation (CV%) of triplicate measurements of free ASN in the flours of twenty-six wheat genotypes from 6 growing environment using the ^{1}H-NMR method was between 19.6% and 26.7%. The range of CV% indicated the existence of relatively large variations in the repeatability of the ^{1}H-NMR technique for concentration quantification purposes. As per Liang et al. (2016), the precision for the measurement of free ASN in leaves of mulberry plants from multiple geographic regions was in the range of 0.77-7.13%

(Liang et al., 2018). As per Claridge (1999), the sensitivity of the ^{1}H-NMR is low compared to many other analytical techniques including chromatography and UV-Vis Spec, and this is considered a drawback of the ^{1}H-NMR instrument (Claridge, 2016). Similar to the sensitivity, high LOQ is another drawback associated with the ^{1}H-NMR technique. In plant and food sciences, the instrument is mainly used for structure determination of metabolites rather than for concentration quantification purposes (Ward et al., 2010). Therefore, this technique is has difficulty detecting compounds of interest at very low concentrations (Claridge, 2016).

2.2.2.3 Enzymatic methods coupled with UV/VIS Spectrophotometer (UV/VIS Spec)

The analysis of L-aspartate and L-asparagine (ASN) by using enzymatic method in one spectrophotometer cuvette was firstly proposed by Williamson in 1962 with the use of L-asparagine amidohydrolase (Bergmeyer, Bernt, Möllering, & Pfleiderer, 1974). In 1972, the use of asparaginase and glutamate dehydrogenase to react with ASN to form ammonia for ASN analysis and quantification was published (Bergmeyer et al., 1974). In recent years, a novel technique that uses a rapid enzyme test kit coupled with UV-Vis Spectroscopy for the analysis of free ASN in food materials was adopted by researchers (Lecart et al., 2018; Shen, Chen, & Li, 2019; Soares et al., 2020). Before the application of the rapid enzyme test kit for analyzing free ASN in whole-wheat flour, deproteinization of sample and pH adjustment of sample solution were two critical procedures (Milman, Cooney, & Applebee, 1978; Sedgwick, Fenton, & Thompson, 1991). Concentrated perchloric acid (PCA) and trichloroacetic acids (TCA) are two types of acids that are commonly used to deproteinize whole-wheat flour sample (Lecart et al., 2018; Milman et al., 1978; Sedgwick et al., 1991). The purpose of sample deproteinization in free amino acid analysis is to precipitate and settle all the protein at the bottom of the sample solution; meanwhile, free amino acids from the whole-wheat flour will remain dissolved in the supernatant to be available for free ASN analysis in subsequent steps (Sedgwick et al., 1991). Concentrated KOH (1M) is usually added to acid-treated solution to adjust the pH of solution to the range of seven to eight, which is the optimal pH levels for the enzymes in subsequent enzymatic reactions (Lecart et al., 2018; Soares et al., 2020).

As per Megazyme (2018), the detection and measurement of free ASN using this novel enzymatic method involves a series of four-step enzymatic reactions by using three types of enzymes:

glutaminase, glutamate dehydrogenase (GIDH), and asparaginase; and two essential chemicals: 2-oxoglutarate and NADPH (Lecart et al., 2018; Megazyme, 2018a). The principles and details of three-step enzymatic reactions for ASN analysis are as follows:

Reaction 1: $L-glutamine+2H_2O \xrightarrow{glutaminase} L-glutamate+NH_4^+$

With the use of the enzyme glutaminase, a stoichiometric reaction takes place to convert glutamine from the whole-wheat flour sample to glutamate and ammonium ion. The purpose of the step is to eliminate the interference by glutamine present in whole-wheat flour sample and to form an ammonium ion for the next reaction (Lecart et al., 2018; Megazyme, 2018a).

Reaction 2: $NH_4^+ + 2-oxoglutarate + NADPH \xrightarrow{GIDH} L-glutamate + NADP^+ + H_2O$

The ammonium ions produced from reaction 1 react with 2-oxoglutarate and NADPH (optimal light absorbance at 340 nm) stoichiometrically to form glutamate and $NADP^+$ (decreased light absorbance at 340 nm) (Lecart et al., 2018; Megazyme, 2018a). The purpose of reaction 2 is to quantify the amount of NADPH that participated in the reaction based on the decrease of absorbance.

Reaction 3: $L-asparagine+2H_2O \xrightarrow{asparaginase} L-aspartate+NH_4^+$

Asparagine forms aspartate and ammonium ion stoichiometrically under the catalyzing effect of asparaginase.

Reaction 4: $NH_4^+ + 2-oxoglutarate + NADPH \xrightarrow{GIDH} L-glutamate + NADP^+ + H_2O$

Ammonium ions produced in step 3 enter reaction 4 to react with the excessive NADPH to form $NADP^+$ and cause a further drop in absorbance (Lecart et al., 2018; Megazyme, 2018a). The purpose of reaction 3 and 4 is to quantify the amount of ASN in the sample based on the decrease of absorbance caused by the formation of ammonium ion that participates into reaction 4.

In terms of quality parameters of the enzymatic method, as per Megazyme Validation Report: L-Asparagine/L-Glutamine/Ammonia Assay Kit (Rapid) (2018), determination of free ASN using the enzymatic reactions has 98.03% accuracy (Megazyme, 2018b). The precision of the enzymatic method is very high with only 0.93% for the coefficient of variation (n=12) (Megazyme, 2018b). In terms of the sensitivity and LOQ, the enzymatic method had better sensitivity and lower LOQ

than the ^{1}H-NMR technique, which allows for quantifying wheat flour free ASN concentrations above 4.9 mg L^{-1} (Megazyme, 2018b).

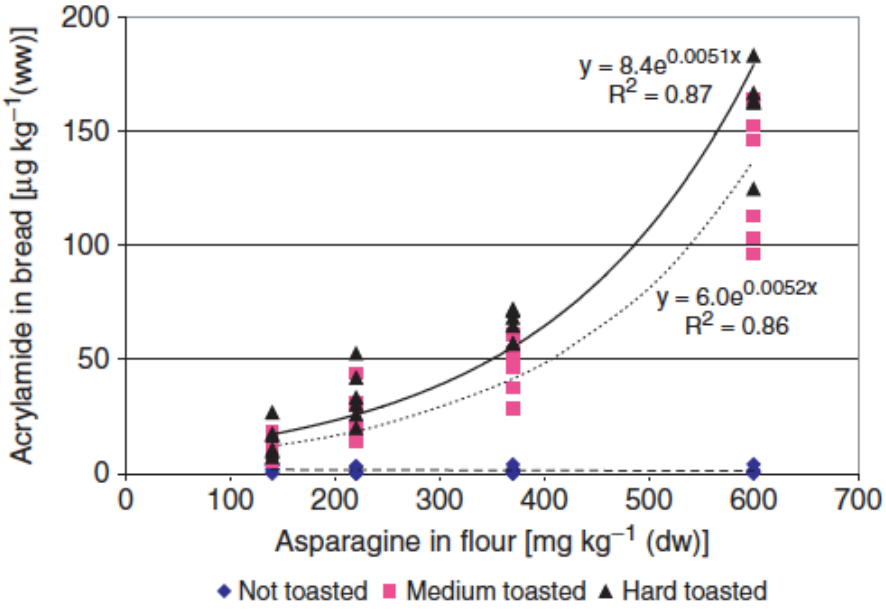
2.3 Relationship between wheat free ASN and bread acrylamide

Free ASN concentration in flour might be a good indication of acrylamide levels in bread (Claus et al., 2006; Granby et al., 2008), since free ASN is the primary precursor of acrylamide formation through the Maillard reaction during the baking process (Mottram et al., 2002; Surdyk et al., 2004). It is meaningful to discover a relationship between ASN in wheat and acrylamide in bread, which can be used to predict the acrylamide levels in bread based on free ASN in the wheat flour. In addition, free ASN concentration in bread dough is also a significant factor to be used to predict acrylamide concentration in bread (Claus et al., 2006). Research groups studied the correlation between free ASN in the flour or dough and acrylamide in the baked breads (Claus et al., 2006; Granby et al., 2008; Hamlet et al., 2008).

Claus et al. (2006) cultivated nine wheat varieties in Ihinger Hof in Germany to study the relationship between free ASN in flour samples and acrylamide levels in breads made thereof. The free ASN in nine wheat genotypes varied from 49.0 to 249.5 mg kg^{-1}, and the resulting acrylamide in bread ranged from 13.8 to 74.4 µg kg^{-1}. A positive linear relationship ($R^2 = 0.75$) was reported between the flour free ASN and the bread acrylamide, indicating that acrylamide formation was raised with elevated free ASN concentrations (Claus et al., 2006b). A few years later, Hamlet et al. (2008) investigated the correlation between free ASN in wheat dough and acrylamide levels in the dough heated at 180 ºC for 20 minutes. They selected bread wheat varieties across the UK with various amounts of free ASN concentrations in flour (207 to 593 mg kg^{-1}), and in bread dough (121 to 325 mg kg^{-1}). The acrylamide concentration in the cooked dough samples under a stimulated baking condition (180 ºC for 20 minutes) ranged from 183 to 436 µg kg^{-1}(Hamlet et al., 2008). A linear relationship ($R^2 = 0.66$) was explored between the free ASN level in the dough and the acrylamide level in the baked dough, which was in agreement with the findings of Claus et al. (2006). However, Granby et al. (2008) came to a result that was different from that of Claus et al. (2006) and Hamlet et al. (2008). Granby et al. (2008) studied the relationship between free ASN in bread wheat flour and acrylamide formation during bread baking and toasting. They prepared bread dough with four levels of free ASN addition (0, 90, 220, and 440 mg kg^{-1}) and baked the

bread at 230 ºC for 2 minutes followed by 30 minutes at 200 ºC (Granby et al., 2008). To analyze the effect of toasting, they measured acrylamide formation in bread after three levels of toasting (none, medium toasted, and hard toasted) on a pushdown toaster. Interestingly, as shown in Figure 2.7, addition of free ASN had no effect on acrylamide formation during baking without a toasting process, due to the low acrylamide levels formed during baking (Granby et al., 2008). However, a significant and direct effect of the addition of free ASN on bread acrylamide levels was observed under medium and hard toasting conditions with $R^2 = 0.86$ and 0.87, respectively (Granby et al., 2008). Granby et al. (2008) suggested that without the toasting process, the acrylamide intake from bread remains low (Granby et al., 2008). Therefore, an insignificant relationship between wheat free ASN and bread acrylamide concentrations might be expected. There could be two main reasons associated with this hypothesis: 1. Without the addition of free ASN in the bread formula, acrylamide formation may be limited when bread dough only undergoes a bread baking process (Granby et al., 2008), 2. Even if acrylamide is formed during the baking process, it might be mainly present in the bread crust (99%), but not the bread crumb due to the nature of the Maillard Reaction (Capuano et al., 2009; Surdyk et al., 2004).

Figure 2.7: Relationship between free ASN levels in wheat flour and acrylamide levels in bread under different toasting conditions. Figure 2a from Granby, K., Nielsen, N. J., Hedegaard, R. V., Christensen, T., Kann, M., & Skibsted, L. H. (2008). Acrylamide–asparagine relationship in baked/toasted wheat and rye breads. *Food additives and Contaminants - Part a Chemistry, Analysis, Control, Exposure and Risk Assessment*, *25*(8), 921–929. doi: 10.1080/02652030801958905. Reprinted of permission from Taylor & Francis journal.



3 Effects of growing environment, genotype, and commercial fertilization levels on free asparagine concentration in Western Canadian wheat

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3.1 Abstract

Background and objectives

Free asparagine (ASN) is the precursor to the formation of acrylamide, which is a probable neurotoxic and carcinogenic compound formed during high temperature (>120 ˚C) processing of starchy foods, such as cereal-based products. Controlling the acrylamide concentration of cereal-based products, e.g., bread, within the allowable levels established by the European Commission is necessary for food safety purposes. One effective measure recommended by the European Commission to mitigate acrylamide in cereal-based products is to reduce free ASN levels in raw ingredients, e.g., wheat. Therefore, knowledge of free ASN levels in Canadian commercial wheat and the strategies to reduce its formation is necessary for the Canadian wheat industry to secure global market access for Canadian wheat. The objective of this study was to understand the effects of genotype, growing environment and fertilization on free ASN concentration of whole-wheat flour from western Canadian wheat varieties.

Findings

The free ASN concentration of whole-wheat flours in this study ranged from 281 to 1014 µg g^{-1} (dry basis). The variation in free ASN levels in wheat was mainly influenced by the growing environment (44%), followed by genotype (31%) and the interaction between genotype and environment (18%). Although being significant, the effects of fertilization and interactions involving fertilization on free ASN concentration in whole-wheat flour were minimal (0.7-2.2%).

Conclusion
Growing wheat genotypes in suitable environments, along with the selection of wheat genotypes with lower potential for free ASN formation are the most effective strategies to control free ASN levels in Canadian wheat.

Significance and novelty
Limited knowledge in regard to free ASN concentration and the strategies to reduce its formation in Canadian wheat may have a serious impact on Canadian wheat access to global markets, especially the European market. This study provides an understanding of the effects of growing environment, genotype, and fertilization on free ASN concentration of select Western Canadian commercial wheat varieties.

Key words:
Free asparagine, Canadian commercial wheat, genotype, growing environment, fertilization

3.2 Introduction

Acrylamide is a naturally generated substance that is commonly found in heat processed carbohydrate-rich foods made from wheat and potatoes, e.g., potato chips and bread (Arvanitoyannis & Dionisopoulou, 2014). Acrylamide is a known carcinogen in animals that may have neurotoxic, mutagenic, and carcinogenic effects on humans (Mottram, Wedzicha, & Dodson, 2002; Stadler et al., 2002; Tareke, Rydberg, Karlsson, Eriksson, & Törnqvist, 2002). Monitoring and preventative food safety measures have therefore been introduced by a number of agencies.

In April 2003, the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) established an international network on acrylamide in food, which was used to share data relevant to the health risk of acrylamide in food (Friedman, 2003a; World Health Organization, 2003). In 2009, Health Canada implemented an acrylamide monitoring program, which was used to evaluate the efficacy of acrylamide reduction strategies. Food products from three classifications were monitored: foods commonly consumed by Canadians, foods with high nutritional values, and foods suspected of having high acrylamide concentration (Health Canada, 2009). In 2017, the Regulation Commission of the

European Union issued acrylamide mitigation measures and recommended benchmark levels for acrylamide in food products, e.g., 50 µg kg^{-1} for wheat bread (European Commission, 2017).

Acrylamide is formed in food through the Maillard reaction of free amino acids and reducing sugars during high temperature (> 120 ºC) processing of starchy foods (Arvanitoyannis & Dionisopoulou, 2014; Mottram et al., 2002; Tareke et al., 2002). Free asparagine (ASN) was found to be the main contributor to the formation of acrylamide in wheat-based products (Mottram et al., 2002; European Commission, 2017). Therefore, reducing the wheat's free ASN is an important approach to controlling acrylamide formation in wheat-based products (Friedman, 2003b; Halford, Muttucumaru, Curtis, & Parry, 2007; P.R. Shewry, Halford, Sodek, Lea, & Parry, 2006).

Numerous research studies have been conducted worldwide on the free ASN concentration in wheat to investigate effective strategies for reducing free asparagine formation in wheat (Corol et al., 2016; Curtis et al., 2009; Malunga et al., 2019; Navrotskyi, Baenziger, Regassa, Guttieri, & Rose, 2018; Ohm, Mergoum, & Simsek, 2017). Selecting the correct wheat genotype, a suitable growing environment with sufficient precipitation and low heat stress, and a proper ratio of nitrogen and sulfur fertilization are three effective approaches to control free ASN concentration in wheat (Corol et al., 2016; Curtis et al., 2009; Granvogl, Wieser, Koehler, Von Tucher, & Schieberle, 2007; Malunga et al., 2019; Navrotskyi et al., 2018; Ohm, Simsek, & Mergoum, 2018; Stockmann et al., 2018; Yadav, Carroll, Estavillo, Rebetzke, & Pogson, 2019).

Different wheat cultivars have exhibited huge variations in free ASN concentration (200 to 1,100 µg g^{-1}) (Navrotskyi et al., 2018). Similar findings were obtained by Malunga et al. (2019) among 30 Canadian hard red spring wheat cultivars: of these Glenn had the lowest free ASN concentration in both wholemeal (302.2 µg g^{-1}) and white flour (116.1 µg g^{-1}), whereas AAC Elie yielded the highest free ASN concentration in wholemeal (700.3 µg g^{-1}) and Muchmore in white flour (335.5 µg g^{-1}) (Malunga et al., 2019).

Growing environment has been shown to have a great impact on the free ASN concentration of wheat (Curtis & Halford, 2016; Martinek et al., 2009; Parisi et al., 2018; Yadav et al., 2019). For example, stresses due to disease, drought, salt in the soil, and solar radiation affect the free

ASN concentration of wheat (Curtis, Bo, Tucker, & Halford, 2018; Navrotskyi et al., 2018; P.R. Shewry et al., 2006). More specifically, stresses related to high temperatures and low precipitation during the grain filling period contribute to the formation of free amino acids (Wilson, Guttieri, Nelson, Fritz, & Tilley, 2020). For example, planting wheat in locations with higher solar radiation leads to an increase in free ASN concentration (Navrotskyi et al., 2018). In addition to solar radiation, crop heat units are also an important factor affecting plant metabolism through heat stress (Corol et al., 2016; Halford, Curtis, Chen, & Huang, 2015). Adequacy of precipitation and moisture retention in the soil are other factors that could influence free ASN accumulation in plants (Gao, Lukow, & Grant, 2012; Giuliani, Giuzio, de Caro, & Flagella, 2011).

Agronomic practices also significantly influence free ASN formation in wheat, especially through the application of nitrogen and sulfur fertilization treatments that result in significant variation in the free ASN concentration of wheat (Curtis et al., 2009; Elmore, Parker, Halford, Muttucumaru, & Mottram, 2008; Halford et al., 2007; Stockmann et al., 2018; Weber, Koller, et al., 2008). Excessive nitrogen and insufficient sulfur in the soil lower protein synthesis efficiency, increasing the tendency for the wheat plant to form non-protein amino acids, including free ASN, glutamine, and aspartate (Halford et al., 2007; P. R. Shewry, Franklin, Parmar, Smith, & Miflin, 1983; P.R. Shewry et al., 2006). Sufficient sulfur (S) concentration in soil was shown to favor the formation of S-rich amino acids (methionine and cysteine) and S-rich storage proteins (α- and γ-gliadin and low molecular weight glutenin) (Peter R. Shewry, Tatham, & Halford, 2001; Steinfurth, Mühling, Zörb, & Albrechts, 2011; Zhao, Hawkesford, & McGrath, 1999). However, low sulfur concentration in soil was reported to favor the formation of S-poor storage proteins (ω-gliadin and high molecular weight subunits of glutenin) and non-protein amino acids, including ASN (P. R. Shewry et al., 1983; Steinfurth et al., 2011; Zhao et al., 1999). Based on observations in Europe, application of sulfur to wheat plants had a greater impact on reducing free ASN accumulation compared to the effect of application of nitrogen on increasing the free ASN concentration in wheat (Curtis et al., 2009; Halford et al., 2007; Peter R. Shewry et al., 2009). However, sulfur fertilization strategies for reducing free ASN concentration in wheat were not always effective. Sulfur fertilization did not affect the formation of free ASN at a wheat grain sulfur content over 0.12% and a nitrogen to sulfur ratio (N/S) in the grain that was less than 17, indicators that the grain has sufficient sulfur (Randall, Spencer, & Freney, 1981; Stockmann et al., 2018; Weber,

Koller, et al., 2008). Sufficient sulfur accumulation in the grains is presumably supplied from the available sulfur in the soil during the grain filling period (Stockmann et al., 2018).

The study of factors affecting free ASN concentration in wheat commercially grown in North America is still very limited (Malunga et al., 2020, 2019; Navrotskyi et al., 2018; Ohm et al., 2017; Wilson et al., 2020). Therefore, the objective of this study was to evaluate the variation of free ASN concentration in select Canadian wheat genotypes grown in different environments under commercially relevant fertilization treatments. A better understanding of approaches to reducing free ASN levels in Canadian wheat genotypes is directly relevant to improving the food safety of grains that are traded internationally.

3.3 Materials and Methods

3.3.1 Wheat and whole-wheat flour samples

Six Canada Western Red Spring varieties (CDC Plentiful, AAC Brandon, AAC Cameron, BW5011, AAC Elie, Glenn), one Canada Prairie Spring Red variety (SY Rowyn), and one Canada Northern Hard Red variety (Prosper) were selected for this study. These eight commercial spring wheat genotypes were grown in four location-years in Manitoba including Carberry (2018), Grosse Isle (2019), and Lilyfield (both years) with three field replicates to allow for an investigation of the effect of growing environment. Four commercial fertilization treatments - two nitrogen levels (100 or 135 kg N ha^{-1}) and two sulfur levels (0 or 17 kg S ha^{-1}) were applied prior to seeding.

At each location, an individual trial was composed of 96 plots in total across 3 replicates (blocks). Within each replicate (block), a split-plot design was adopted with four fertilization treatments as main plots and eight varieties as sub-plots randomized within each main plot. Fertility treatments were also randomized within each replicate. During harvest, wheat grains from the three field replicates were combined into one composite grain sample, i.e., 32 composite treatments from each location-year, resulting in 128 treatments in total with each treatment being analyzed in triplicate in the laboratory.

Harvested wheat grains were cleaned, tempered to 16.5% for 24 hours, and milled into straight grade flour. Grain was milled to pass through a 150 µm sieve at a 75% extraction rate using a

Bühler laboratory flour mill (MLU-202) with a Bühler impact finisher (MLU-302). To prepare whole-wheat flour for ASN analysis, the bran portion from the Bühler mill was milled with a Jacobson hammer mill to pass through a 1190 µm screen. This milled bran was added to the straight grade flour from the Bühler mill.

3.3.2 Chemicals and reagents

L-ASN and L-norvaline standards were obtained from Sigma-Aldrich (Milwaukee, WI, USA). AccQ•Tag Ultra derivatization kit and AccQ•Tag Eluents A and B were purchased from Waters Limited (Mississauga, ON, Canada). UPLC grade hydrochloride, acetonitrile, and filtration membranes were acquired from Fisher Scientific (Whitby, ON, Canada). Water was deionized and then filtered with a 0.2 µm Millipore filter membrane purchased from Fisher Scientific (Whitby, ON, Canada).

3.3.3 Analysis

Weather data was collected from Manitoba Ag-Weather Program growing season reports (2018, 2019) from Manitoba Agriculture, Food and Rural Development. Soil samples were collected at two depths including 0-15 cm and 15-60 cm. The nitrate-nitrogen content was quantified by a cadmium reduction method, and sulfur content was determined using a barium chloride turbidimetric method (Malunga et al., 2020).

The moisture content of whole-wheat flour was determined according to AACCI Method 44-15.02 prior to free ASN analysis. The free amino acids from whole-wheat flour (200 mg) were extracted with 0.01 M hydrochloric acid solution and derivatized with a AccQ•Tag Ultra pre-column derivatization amino acid kit to form stable urea for UPLC analysis as previously reported (Malunga et al., 2019). The internal standard L-norvaline (250 µM) was added into the 0.01 M hydrochloric acid solution and incorporated into each sample during extraction to improve the precision of free ASN quantification. UPLC analyses were performed using a Waters H class ultrahigh pressure liquid chromatograph, equipped with quaternary solvent manager, sample manager, heated column compartment, and photodiode array detector (Waters, Mississauga, ON,

Canada) as per Malunga et al. (2019). A Waters AccQ•Tag Ultra C_{18} column (2.1x100mm) was used for the UPLC separation of extracted components.

3.3.4 Statistical analysis

JMP Pro 14 statistical software (SAS Institute Inc., Cary, NC) was used to determine the effects of genotype, environment, fertilization, and the interactions between these factors, i.e., genotype-environment, genotype-fertilization, environment-fertilization and genotype-environment-fertilization. Before adopting a Mixed model analysis, the normality of the data distribution was checked. Data were subject to a three-way analysis of variance (ANOVA) with environment, genotype, fertilization, and their interactions as fixed factors. Pairwise comparisons were conducted for treatment means (n=3) by the Tukey method to determine significance at $P < 0.05$. The Pearson correlation test was used to determine the relationship between the ratio of free ASN concentration to total grain protein versus grain yield at a significance level of $P < 0.05$.

3.4 Results and Discussion

3.4.1 Free ASN concentration and source of variation

Similar to studies conducted in Europe, there was considerable variation in free ASN among wheat samples, and this was strongly associated with genotype, growing environment, and agronomic practices (Corol et al., 2016; Curtis et al., 2009; Stockmann et al., 2018; Weber, Koller, et al., 2008). The analysis of variance results (Table 3.1) indicated that growing environment (E) predominated (44.3%) the total variance in the measured free ASN in whole-wheat flour samples, followed by genotype (G), and then by the interaction between environment and genotype (G×E), contributing 31.2% and 18.2%, respectively. For the wheat samples examined in this study, grown under commercial conditions, fertilization (F) had the least impact on free ASN concentration, contributing only 0.7% to the variance in ASN concentration. Interactions between genotype and fertilization (G×F) and the interaction between environment and fertilization (E×F) contributed to only 1.4% and 0.4% of the total variance, respectively.

The role of environment as the most significant factor affecting free ASN formation in wheat was also reported by Ohm et al. (2017), who reported that growing location contributed the most to the

total variance (at 32.5%) in the free ASN concentration in a study on 75 hard red spring wheat genotypes grown in three locations in one crop year in North Dakota. Genotype and the interaction between genotype and location accounted for 26.6% and 4.6% of the total variance, respectively.

Table 3. 1: Three-way ANOVA showing the influence of genotype, environment, fertilization, and their interactions on the free asparagine concentration of whole-wheat flour.

Type III Tests of Fixed Effects					
Effect	Degrees of freedom	Type III SS	F Value	Pr > F	Percent of total variance
Environment (location-year) (E)	3	4655724.58	2426.85	< 0.0001	44.3%
Genotype (G)	7	3287841.59	734.50	< 0.0001	31.2%
Fertilization (F)	3	71704.82	37.38	< 0.0001	0.7%
G×E	21	1919908.16	142.97	< 0.0001	18.2%
G×F	21	147547.52	10.99	< 0.0001	1.4%
E×F	9	40238.31	6.99	< 0.0001	0.4%
G×E×F	63	234624.59	5.82	< 0.0001	2.2%
Error	256	163705.25	N/A	N/A	1.6%

3.4.2 Effect of environment

The growing environment contributed the most to free ASN concentration of whole-wheat flour (Table 3.1). For the growing environments (location-years) studied there was significant variability in average ASN concentration as well as the range in ASN concentration at a given location-year. In terms of ASN concentration distribution, Carberry in 2018 had the widest range for free ASN (397-1057 µg g^{-1}), followed by Lilyfield in 2019 (414-914 µg g^{-1}), Lilyfield in 2018 (269-696 µg g^{-1}) and Grosse Isle in 2019 (306-579 µg g^{-1}).

Comparing the free ASN concentration of two locations in each year, it was found that in 2018, wheat genotypes grown in Carberry had on average over 40% higher free ASN than those grown in Lilyfield, an outcome that could arise from one or two mechanisms. Firstly, Carberry had longer harvest period and over 20% higher crop heat units than Lilyfield in 2018 (Table 3.2), indicating

that the wheat plots in Carberry were exposed to more cumulative heat over the growing season from seeding to harvest. Similar results were reported by other researchers, e.g., heat stress led to higher free ASN accumulation in wheat crops (Corol et al., 2016; Halford et al., 2015). Secondly, Carberry has a Wellwood Orthic Black Chernozem type of soil, which is a sandy-like soil known to be prone to moisture loss (Fuller, 2010), while Lilyfield has Red River Clay soil which is excellent at retaining moisture (Kandel et al., 2013). Free ASN tends to accumulate in the grain to a larger extent when wheat plants are subject to drought stress (Navrotskyi et al., 2018; Ohm et al., 2017). Both reasons led to a tendency to a greater degree of free ASN formation and accumulation in wheat crops grown at Carberry compared to those grown at Lilyfield.

In 2019, the same genotypes grown at Lilyfield had on average almost 60% higher free ASN than those grown at Grosse Isle. There were two mechanisms affecting free ASN concentration in wheat grains. First, Lilyfield had longer harvest days and 7% higher crop heat units than Grosse Isle in 2019 (Table 3.2), so that the wheat plots grown in Lilyfield were subject to higher heat accumulation over the growing season (Corol et al., 2016; Halford et al., 2015). Second, the Dark Gray Chernozem soil at Grosse Isle has less water retention capacity compared to the Red River Clay soil at Lilyfield, promoting higher free ASN levels in wheat grown at Grosse Isle (Fuller, 2010; Kandel et al., 2013). However, the results suggested that the role of crop heat unit was dominant over the role of soil type in free ASN accumulation in hard red spring wheat cultivars of this study in 2019. A comparison between the crop years 2018 and 2019 for Lilyfield indicated that the wheat grown in 2019 had 50% higher free ASN concentration compared to wheat grown in 2018, which could be attributable to two mechanisms. Firstly, the soil N/S ratios in 2018 were 0.82 and 0.32 at two depth levels, while they were 1.10 and 0.59 in 2019 (Table 3.2). Therefore, wheat plants in 2019 were exposed to more nitrogen and less sulfur than wheat crops grown in 2018. This 50 - 80% larger N/S ratio in 2019 could be the reason for a higher ASN concentration of 2019 wheat crops compared to 2018 wheat crops (Curtis et al., 2009; Halford et al., 2007). For 2019, the total growing days increased by 30% compared to 2018. This indicated that differences in growing conditions, such as growing days, soil type, temperature, and precipitation led to different responses of the crops in terms of their free ASN concentration, as was previously demonstrated in both North America and Europe (Navrotskyi et al., 2018; Ohm et al., 2017; P.R. Shewry et al., 2006; Wilson et al., 2020).

Figure 3.1: Free ASN concentration (µg g^{-1}) of wheat grown in four location-years (Carberry 2018, Lilyfield 2018, Grosse isle 2019, and Lilyfield 2019) under four fertilization treatments (100 kg N ha^{-1}, 100 kg N ha^{-1} + 17 kg S ha^{-1}, 135 kg N ha^{-1}, and 135 kg N ha^{-1} + 17 kg S ha^{-1}).

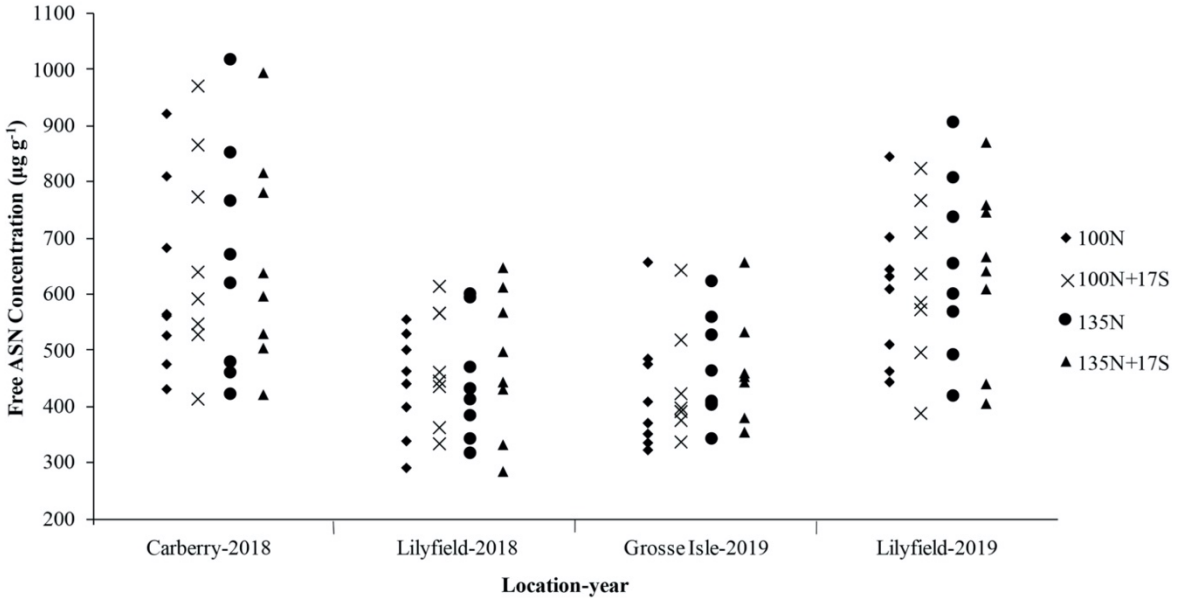


Table 3. 2: Meteorological and soil information of the four growing environments (location-year)

Crop year	Location	Weather Station	Soil Test						Soil Type	Days to Harvest	Crop Heat Units	Total Precipitation (mm)
			0-15 cm		N/S Ratio	15-60 cm		N/S Ratio				
			N (kg ha^{-1})	S (kg ha^{-1})		N (kg ha^{-1})	S (kg ha^{-1})					
2018	Carberry	Carberry	24.7	16.8	1.47	201.8	65	3.1	Wellwood Orthic Black Chernozem	115	2467	235
	Lilyfield	Winnipeg Airport	14.6	17.9	0.82	11.2	34.8	0.32	Red River Clay	92	2044	230
2019	Grosse Isle	Woodlands	17.9	13.5	1.33	57.7	45.4	1.27	Dark Gray Chernozem	113	2174	204
	Lilyfield	Winnipeg Airport	17.9	16.3	1.10	35.9	60.5	0.59	Red River Clay	120	2323	223

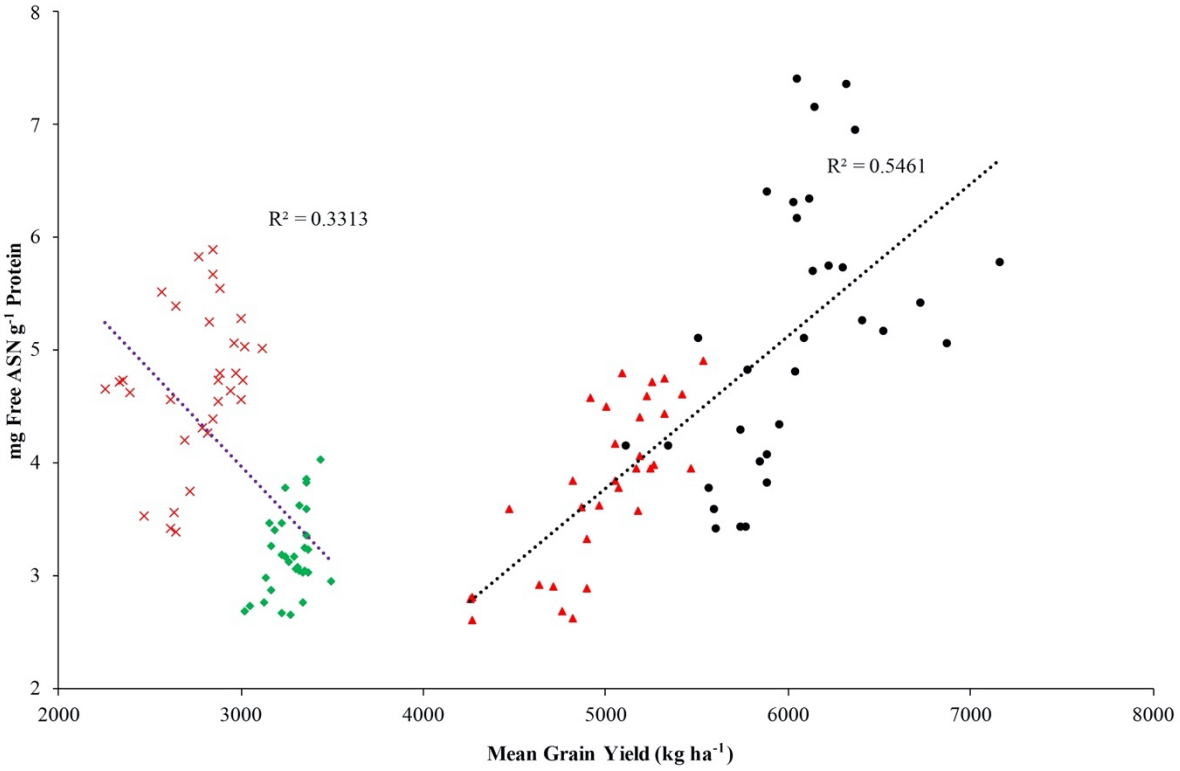
Table 3. 3 : Grain yield information of the four growing environments (location year)

Crop year	Location	Mean Grain Yield (kg ha^{-1})	Mean Grain Protein Content (%)	Mean Free ASN (µg g^{-1})	Mean Free ASN Per Unit of Protein (mg g^{-1})
2018	Carberry	6034	14.0	651	4.6
	Lilyfield	4990	13.8	457	3.3
2019	Grosse Isle	3274	15.4	414	2.7
	Lilyfield	2759	15.6	657	4.2

Comparing the two crop years 2018 and 2019, precipitation in the latter was much lower than the former, which resulted in a significant drop in mean grain yield in 2019 (Table 3.3). The lower mean grain yield in 2019 was probably associated with less water available in the soil, causing water deficiency in the plant. A similar trend was observed by Mkhabela et al. (2010), where grain yield was negatively affected by the water demand of wheat plants (Mkhabela, Bullock, Gervais, Finlay, & Sapirstein, 2010). Water stress, especially the availability of water between July and August, had a huge impact on spring wheat grain yield (Qian, De Jong, Warren, Chipanshi, & Hill, 2009). Precipitation affects not only the grain yield but also the grain protein content (Jarvis et al., 2008; Johansson & Svensson, 1997; Mkhabela et al., 2010; Sattar et al., 2020; Smith & Gooding, 1996). In this study, the low precipitation in 2019 resulted in higher protein content in the wheat grains (Table 3.3). Water stress has been cited as a reason for increasing protein content in grain (Sattar et al., 2020; Smith & Gooding, 1996).

When comparing the relationship between the ratio of free ASN concentration to total grain protein versus mean grain yield in the two crop years (2018 and 2019), two contrasting trends were observed. In 2018, a positive correlation was found (R = 0.739), whereas a negative correlation was seen for 2019 (R = -0.575) (Figure 3.2). This means, that in 2019 (Figure 3.2), when the grain yielded less, the proportion of free ASN remaining in the grain decreased as the yield increased. One probable mechanism is that water stress limited the formation of excessive free ASN in grains; due to the activity of asparaginase in the grain, asparagine was catabolized to release nitrogen for the synthesis of other amino acids (Curtis et al., 2018; Sotero-Martins, Da Silva Bon, & Carvajal, 2003). However, in the year 2018 when a lot more moisture was available, the correlations were the opposite. In 2018, higher grain yields led to an increase in the relative amount of free ASN as yield increased. It should be noted that the trends in Figure 3.2 were also evident for free ASN concentration against yield, but R^2 values were lower than on a unit protein basis.

Figure 3.2: Relationship between the proportion of free ASN concentration relative to total grain protein content against mean grain yield for wheat grown in four environments (location-year); in 2018 at Carberry (●) and Lilyfield (▲), and in 2019 at Grosse isle (♦) and Lilyfield (×).

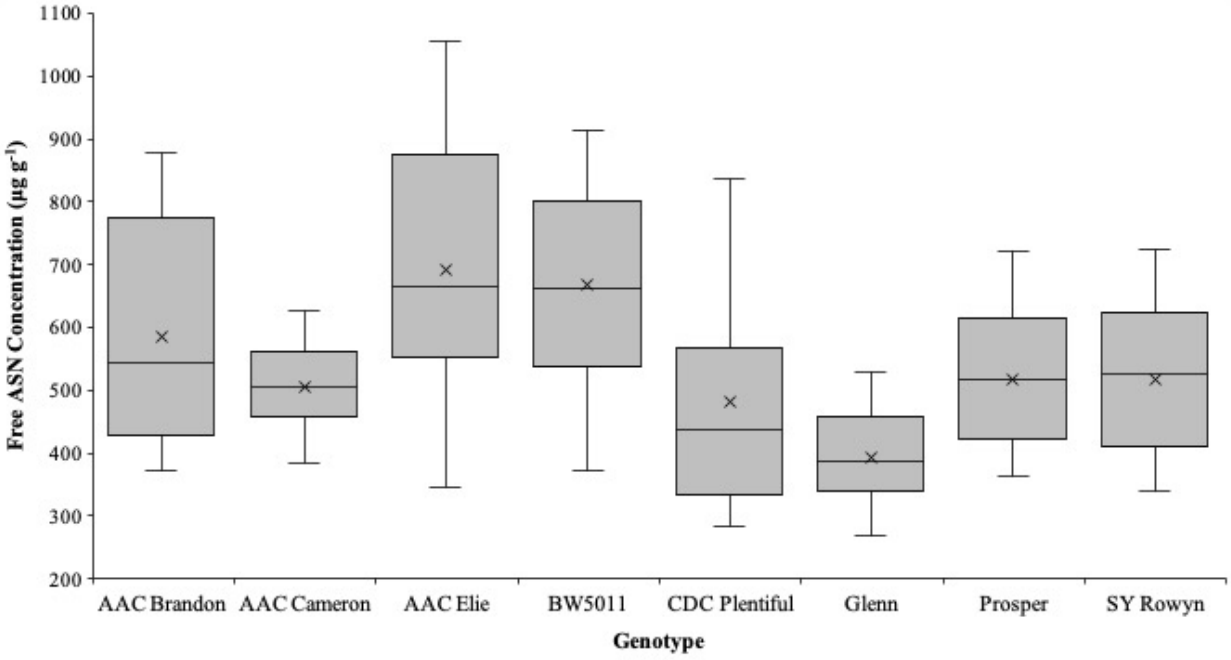


3.4.3 Effect of genotype

Significant variation in the free ASN concentration of whole-wheat flour was strongly associated with wheat genotype ($p < 0.0001$). Among the eight genotypes studied, Glenn had the lowest mean value (393 µg g^{-1}) and AAC Elie had the highest (690 µg g^{-1}), but considerable variation over all treatments was apparent (Figure 3.3). A number of studies have shown, both in the US and Canada, that Glenn is a genotype that accumulates low free ASN (Li, Tyl, Kaiser, & Annor, 2019; Malunga et al., 2019; Ohm et al., 2017). It was reported that Glenn had good performance in terms of baking results and consistently low free ASN in all growing locations (Liu, Ohm, Hareland, Wiersma, & Kaiser, 2011; Ohm et al., 2017). AAC Cameron and Glenn had a narrow range of free ASN concentration over all treatments compared to the other genotypes, which indicated that the formation of free ASN in these two varieties varied less in response to different growing environments and different nitrogen and sulfur fertilization treatments. In contrast, AAC

Elie had a very wide range in free ASN concentration, indicating that this genotype is very susceptible to changes in growing environment or fertilization (Figure 3.3).

Figure 3.3: Free ASN concentration (µg g^{-1}) of eight Canadian hard red spring wheat genotypes averaged over four grown location-years (Carberry 2018, Lilyfield 2018, Grosse isle 2019, and Lilyfield 2019) under four fertilization treatments (100 kg N ha^{-1}, 100 kg N ha^{-1} + 17 kg S ha^{-1}, 135 kg N ha^{-1}, and 135 kg N ha^{-1} + 17 kg S ha^{-1}).



3.4.4 Effect of fertilization

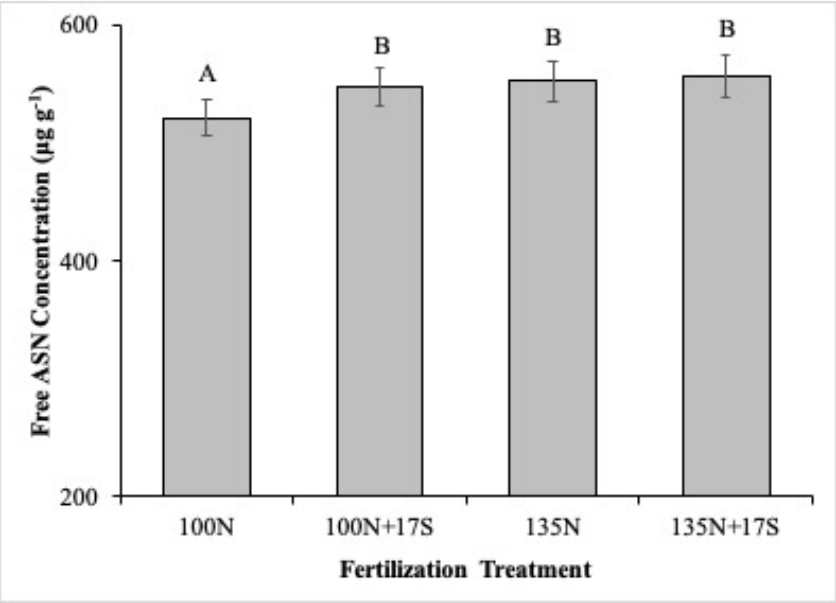
Fertilization resulted in significant differences in free ASN concentration in whole-wheat flour samples ($p < 0.0001$). However, the effect of fertilization on free ASN variations was less pronounced compared to the effects of environment and genotype, due to less variation in mean free ASN levels associated with the four commercial fertilization treatments. It was found that wheat grown under 100 kg N ha^{-1} fertilizer yielded the lowest mean free ASN concentration (521 µg g^{-1}). The application of sulfur on top of the same nitrogen level (100 kg N ha^{-1} + 17 kg S ha^{-1}) and increasing the nitrogen fertilizer level (135 kg N ha^{-1}) resulted in higher free ASN concentrations. The highest mean concentration of free ASN was found in whole-wheat flour samples from crops grown under 135 kg N ha^{-1} + 17 kg S ha^{-1}, at 557 µg g^{-1} (Figure 3.4). Even

though the overall range of wheat free ASN concentration over four fertilization treatments was relatively narrow, i.e., 521-557 μg g^{-1}, these results followed the trend reported in studies where much wider ranges in nitrogen were applied: increasing nitrogen application led to higher free ASN concentrations (Martinek et al., 2009; Weber, Graeff, et al., 2008; Weber, Koller, et al., 2008). For example, an increase in nitrogen fertilizer from 0 to 180 kg N ha^{-1} led to a 250% increase in free ASN in wheat (Martinek et al., 2009).

However, the effect of sulfur application on free ASN concentration of wheat was not consistent throughout this study. For example, in crop year 2018 at Lilyfield, adding 17 kg S ha^{-1} increased free ASN concentration of wheat grains regardless of nitrogen fertilization levels, whereas in crop year 2019 at Grosse Isle, adding 17 kg S ha^{-1} on top of 135 kg N ha^{-1} reduced free ASN concentration of the grains. In sulfur-deficient soils, the addition of sulfur fertilizer resulted in a significant decrease in free ASN concentration of wheat (Curtis et al., 2009; Muttucumaru et al., 2006; P. R. Shewry et al., 1983; P.R. Shewry et al., 2006; Peter R. Shewry et al., 2009; Wilson et al., 2020). There were no beneficial effects related to the application of sulfur to our wheat plants, which could be due to the naturally sufficient sulfur levels in the soil at the locations utilized in this study. In all four growing environments, the N/S ratio was lower than 17 (Table 3.1), which indicated an adequate supply of sulfur from the soil to the wheat plants (Randall et al., 1981; Stockmann et al., 2018; Weber, Koller, et al., 2008). Thus, the addition of sulfur fertilizer did not lead to a significant reduction of free ASN concentration in the wheat grain. A similar result was obtained in Europe, where sulfur fertilization during conventional farming had no significant influence on free ASN concentration in wheat as compared to organic farming (Stockmann et al., 2018; Weber, Koller, et al., 2008).

Thus, in this situation, application of 100 kg N ha^{-1} fertilization would be recommended to lower the free ASN of hard red spring wheat. However, incorporation of sulfur is not recommended due to no overall impact on free ASN reduction in wheat in these relatively sulfur-rich soils.

Figure 3.4: Mean free ASN concentration (µg g^{-1}) of 8 wheat varieties grown at 4 location-years (Carberry 2018, Lilyfield 2018, Grosse Isle 2019, and Lilyfield 2019) subject to four fertilization treatment (100 kg N ha^{-1}, 100 kg N ha^{-1} + 17 kg S ha^{-1}, 135 kg N ha^{-1}, and 135 kg N ha^{-1} + 17 kg S ha^{-1}).

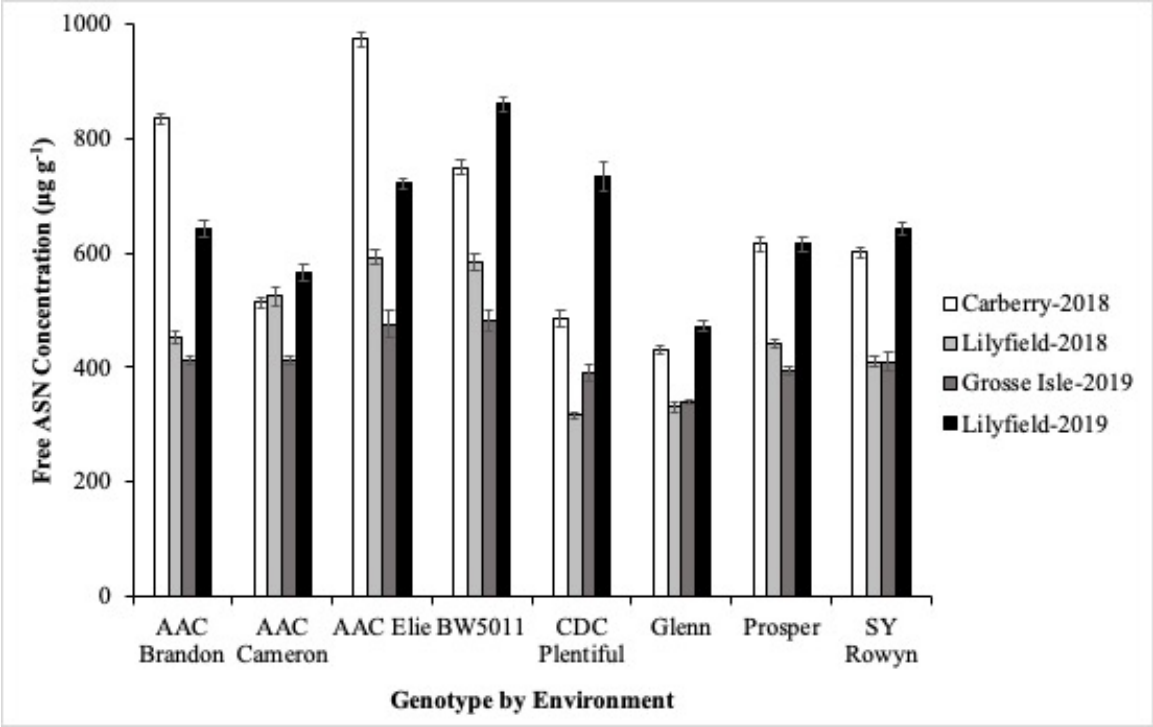


3.4.5 Interactions between environment, genotype, and fertilization

The level of effects due to interactions was found to be lower than the two predominant individual factors of environment and genotype (Table 3.1). A comparison of F values from ANOVA indicated that the interaction between E*G affected the free ASN concentration of whole-wheat flour samples to a much larger extent compared to the other interactions. A significant effect of E*G on free ASN concentration indicated that wheat genotypes responded differently to location-year in terms of their free ASN concentration (F = 142.96). For example, in 2018, AAC Elie grown at Carberry had the highest free ASN concentration among all genotypes (974 µg g^{-1}), whereas, in 2019, the highest free ASN concentration was observed in BW5011 grown at Lilyfield (860 µg g^{-1}) (Figure 3.5). Moreover, in each site-year, AAC Brandon and AAC Elie yielded the highest free ASN levels in Carberry 2018, whereas BW5011 and CDC Plentiful accumulated the highest free ASN concentrations in Lilyfield 2019. These observations indicate that wheat genotypes responded differently to the growing environment in terms of asparagine accumulation.

The G*F interaction also had a significant effect on the free ASN concentration of wheat varieties (Table 3.1). Genotypes responded differently, in terms of their ASN concentration, to fertilization treatment. For example, the effect of fertilization on free ASN concentration of AAC Cameron was significant while the free ASN concentration of AAC Brandon did not change significantly in response to the application of different nitrogen or sulfur fertilizers. Therefore, wheat genotype should be taken into consideration when selecting fertilization treatments to reduce free ASN levels in the grain. As noted above, higher nitrogen applications increased ASN concentrations. For the varieties AAC Elie, BW5011 and Plentiful, increased concentration of ASN was observed at each of the 4 location-years when the nitrogen rate was increased from 100 to 135 kg N ha^{-1}, such that there was on average a 12.7% increase in ASN concentration. The application of sulfur to the 135 kg N ha^{-1} treatment eliminated this increase for AAC Elie, but BW5011 and Plentiful did not respond to the applied S.

Figure 3.5: Free ASN concentration (µg g^{-1}) of eight wheat genotypes grown in four location-years (Carberry 2018, Lilyfield 2018, Grosse Isle 2019, and Lilyfield 2019) averaged over four fertilization treatments (100 kg N ha^{-1}, 100 kg N ha^{-1} + 17 kg S ha^{-1}, 135 kg N ha^{-1}, and 135 kg N ha^{-1} + 17 kg S ha^{-1}).



3.5 Conclusions

The free ASN concentration in eight Canadian commercial hard red spring wheat genotypes was significantly affected by growing environment, genotype, and fertilization as well as the interaction terms E*G and G*F. Environment effects (location-year) contributed to the largest portion of the variation in free ASN in wheat, followed by genotype, which much lesser effect of fertilization. Selection of wheat genotypes is important though, since some varieties had consistently low ASN levels regardless of environment. A careful administration of fertilization treatments is needed in order to control free ASN levels in Canadian wheat. Specifically, commercial application levels of sulfur fertilization are not recommended since in the locations studied, where soil sulfur was not deficient, no significant effect of sulfur application on free ASN levels was observed.

3.6 Acknowledgment

This project was made possible due to funding from the Governments of Manitoba and Canada through the Canadian Agricultural Partnership as well as funding received from Warburtons, Manitoba Wheat and Barley Growers Association, Saskatchewan Wheat Development Commission, Alberta Wheat Commission, SeCan and FP Genetics. We are thankful to Paterson Grain for growing wheat samples of this study. Technical support from milling and analytical services departments of the Canadian International Grains Institute is also acknowledged. We specially thank members of Drs. Ames and Joseph's laboratory at Agriculture and Agri-Food Canada for their technical support. No funding or material support for this study led to conflict of interest in the preparation and publication of this manuscript.

Connection between Chapter 3 and 4

The knowledge gained in Chapter 3 gives a perspective on the causative factors related to acrylamide food safety concerns in bakery products. There is a need to understand how genotype, growing environment, and agronomic practices affect the free asparagine concentration in western Canadian wheat so that strategies for free asparagine reduction in Canadian wheat can be implemented to prevent market access restrictions due to the presence of ASN as the precursor to acrylamide. For example, selecting varieties with low free asparagine concentration and growing the wheat under a commercial level of nitrogen fertilization are two recommended solutions. With the emerging demand of assessing the free asparagine concentration in the global wheat trade, a method of free asparagine analysis that is able to deliver rapid results with high quality using easily accessible experiment materials and instruments is necessary. The method should be applicable in simple laboratory environments or even at port for screening cargo shipments for their free asparagine concentration in wheat. Therefore, in the following chapter (Chapter 4), a rapid enzymatic test method was evaluated and compared against two other established techniques for free asparagine analysis - Ultrahigh Performance Liquid Chromatography (UPLC) and Proton Nuclear Magnetic Resonance (^{1}H-NMR). A comprehensive comparison is carried out using various quality parameters to help understand the performance of the rapid enzymatic test method.

4 Comparison between a rapid analytical method and two well developed methods for wheat free asparagine detection and quantification

Author

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4.1 Abstract

Background and objectives

To fulfill the emerging demand for free asparagine (ASN) analysis due to acrylamide-related food safety issues in baked cereal products, a rapid, user-friendly method that can provide reliable results without requiring complicated sample preparation and sophisticated instrumentation is necessary. This study validated a novel approach of using a new rapid enzymatic test for wheat free asparagine analysis by comparing this new method with two established analytical methods: UPLC and ^{1}H-NMR using ASN standards and a range of free ASN concentrations extracted from whole-wheat flours obtained from wheat (a range of varieties and locations). For the new method validation, analyses were performed to assess the quality parameters for all three methods, including accuracy, precision, and sensitivity using solutions of asparagine. Also, the experimental and time costs associated with the analyses were estimated.

Findings

The rapid test method had good and consistent accuracy of 93.2 to 93.5 % and excellent precision of 1.02 and 0.44% (CV%) for two reference solutions with concentrations of 15 and 60 mg L^{-1} L-ASN. Also, the rapid test method had excellent linearity for its calibration curve with Pearson's correlation r = 0.9916 and good sensitivity of 0.0389 AU L mg^{-1} in detecting the free ASN signal using a UV/VIS Spectrophotometer. Cost-wise, the rapid test method represented the least cost for experimental materials and instrument usage and took about 40-50 minutes to generate one free ASN concentration result. Very strong correlations of free ASN concentration in whole-wheat flour were found for the results generated by the rapid test method and the UPLC and the ^{1}H-NMR method with the Pearson's correlation r of 0.9970 and 0.9081, respectively.

Conclusion
Overall, based on the comprehensive assessment in this study, the rapid test method is recommended for ASN analysis in wheat grains in simple laboratory environments or industrial applications.

Significance and novelty
This is the first study that provides a comprehensive summary of the performance of three different free ASN analytical methods, including accuracy, repeatability, sensitivity, and costs. It also assessed the relationships between free ASN concentration in wheat measured by the new rapid test with the UPLC and ^{1}H-NMR techniques.

Key words:
Free asparagine, analytical chemistry methods, UPLC, ^{1}H-NMR, Megazyme

4.2 Introduction

Great attention has been paid to acrylamide (CH_2=CH-CO-NH_2) by researchers around the world (Amrein, Schönbächler, Escher, & Amadò, 2004; Capuano et al., 2009; Curtis & Halford, 2016a; Ohm et al., 2017), after acrylamide was identified as a class 2A carcinogen for humans by the International Agency for Research on Cancer (IARC) in 1994 (International Agency for Research on Cancer, 1994; JEFCA, 2005). In wheat plants and grains, free asparagine (ASN) is the main precursor in the Maillard reaction, determining the amount of acrylamide formed in wheat products (Halford et al., 2007; Weber, Graeff, et al., 2008). Free ASN is relevant to acrylamide formation in baked products, such as bread, cake, and biscuit (Mottram et al., 2002; Stadler et al., 2002) because in the Maillard reaction free ASN reacts with reducing sugars during high temperature (beyond 120 ºC) processes that involve surface dehydration of bread dough or cake batter. ASN (H_2N-CO-CH_2-CH(NH_2)-COOH) has a molecular mass of 132.32 g mol^{-1} and contains an amino group and a carboxylic group. As one of the most dominant non-essential amino acids to humans that is formed in the wheat plant, free ASN makes up to 27 % of the total free amino acid pool in wheat grains grown under a sulfur sufficient condition, but up to 50 % when wheat is grown under severe sulfur deficient conditions (Curtis et al., 2009; Muttucumaru et al., 2008).

Measurement of free ASN concentration in wheat is therefore critical for evaluation of the acrylamide forming potential of a wheat flour that is to be processed into bakery products. Various techniques have been developed for free ASN detection and quantification in different cereal grains by different research groups (Table 4.1). Among the methods, chromatography techniques (liquid and gas chromatography) are the most popular. Chromatography techniques have been adopted by many researchers worldwide for free amino acid analysis since almost two decades ago (Curtis et al., 2009; Curtis, Powers, et al., 2018; Granby et al., 2008; Granvogl et al., 2007; Muttucumaru et al., 2006a) and they are still the most widely used reliable method at present (Malunga et al., 2021; Xie et al., 2021). In particular, ultra-high-performance liquid chromatography (UPLC) equipped with photodiode array detector (PDA) has been utilized in recent years for detection and quantification of free ASN in wheat (Çelik & Gökmen, 2020; Malunga et al., 2019; Xie et al., 2021). Nuclear Magnetic Resonance (NMR) is a well-established advanced, non-destructive technique that has been used to identify molecular structure, bonds, and interactions of compounds of interest in agriculture and food science applications (Claridge, 2016; Hore, 2015). NMR has therefore been used to detect and quantify plant metabolites, and food components such as lipids, carbohydrates, polysaccharides, and amino acids (Hatzakis, 2019). Proton Nuclear Magnetic Resonance (^{1}H-NMR) is a relatively novel analytical chemistry technique that has been applied in food science since the end of 20th century (Hatzakis, 2019). Its application for free ASN analysis became popular since 2016 when introduced by Corol et al. (Corol et al., 2016). Each analytical chemistry analysis technique comes with its strengths and drawbacks, which also applies to these two techniques. A strong dependence on the availability of laboratory resources and proficient experimental skills are common drawbacks limiting the use of both chromatography and NMR methods.

Table 4.1: Summary of analytical methods for free ASN detection and quantification in cereal or processed cereal products

Technique	Detector	Food material	Sample preparation	Internal Standard	Instrument/ column	Method parameters	CV %	Reference
UPLC	Photodiode array detector (PDA)	Whole-wheat flour	Sample milled, free amino acid extraction with 0.01 M hydrochloric acid (HCl), and Waters AccQ-tag derivatization	Norvaline	H-class UPLC with Waters AccQ-Tag Ultra C18 column (2.1 x 100 mm)	0.1 % formic acid in acetonitrile, 10 % acetonitrile in water, water, and acetonitrile, for solvent A, B, C, and D, respectively Flow rate 0.7 mL min^{-1}	0.24-9.91	Xie et al., 2021
UPLC	PDA	Ground wheat wholemeal and wheat flour	Sample ground, free amino acid extraction with 0.01 M HCl, and Waters AccQ-tag derivatization	None	H-class UPLC with Waters AccQ-Tag Ultra C18 column (2.1 x 100 mm)	0.1 % formic acid in acetonitrile, 10 % acetonitrile in water, water, and acetonitrile, for solvent A, B, C, and D, respectively Flow rate 0.7 mL min^{-1}	0.24-9.91	(Malunga et al., 2019)
HPLC	Fluorescence detector (FLD)	Wholegrain flour of Wheat, rye, and spelt	Sample milled, free amino acid extraction with 45 % ethanol , and FMOC derivatization	None	LiChroCART Superspher RP 8 column (250 mm x 4 mm)	Constant temperature at 45 ºC, fluorescence intensity measured at 263 and 313 nm	n/a	(Žilić et al., 2017)
HPLC	FLD	Wheat flour and wholegrain flour of wheat, durum wheat, oat, rye, barley, and maize	Sample milled, free amino acid extraction with water, and o-phtaldialdehyde derivatization	None	LiChroCART Superspher RP 8 column (250 mm x 4 mm)	50 mM potassium phosphate buffer, acetonitrile, and tetrahydrofuran (82.5:14.5:3) solvent Flow rate: 1.0 mL min^{-1}	26.83-54.17	(Žilić, Aktağ, Dodig, Filipović, & Gökmen, 2020)
HPLC	FLD	Wheat flour, rye flour, and spelt flour	Sample milled, free amino acid extraction with 45 % ethanol , and FMOC derivatization	None	LiChroCART Superspher RP 8 column (250 mm x 4 mm)	Constant temperature at 45 ºC, fluorescence intensity measured at 263 and 313 nm	n/a	Stockmann, Weber, Mast, et al., 2019; Stockmann, Weber, Merkt, et al., 2019;
HPLC	FLD	Wheat flour	Sample milled, free amino acid extraction with 50 % methanol and 0.005 M HCl, and o-phthaldialdehyde derivatization	Homoserine	Information not available (n/a)	n/a	n/a	Martinek et al., 2009
HPLC	LC-240 FLD	Ground wheat wholemeal, and white flour	Sample ground, free amino acid extraction with 0.01 M HCl, and OPA with 3- mercaptopropionic acid derivatization	None	ZORBAX Eclipse-AAA (Agilent) column	0.02 M phosphate buffer, water, and acetonitrile/ methanol/ water, for solvent A and B, respectively. Flow rate 0.7 mL min^{-1}	9.2	Hamlet, Sadd, & Liang, 2008
LC	Tandem Mass Spectrometry (MS/MS)	Wheat flour and rye flour	Free ASN extraction with MilliQ water, followed with solid phase extraction cleanup using LiChroLut RP-C18 solid phase extraction (SPE) cartridge	^{15}N-asparagine	Hypercarb LC column (2.1 x 100 mm)	0.1 % formic acid solvent Flow rate: 0.2 mL min^{-1}	0-18	Granby et al., 2008

GC	Flame ionization detection (FID)	Wheat flour, rye flour, and spelt flour	Free ASN extraction with 45 % ethanol, followed with EZ-Faast cleanup, and chloroform/2,2,4-trimethylpentane derivatization	Norvaline	Zebron-AAA column with an HP 6890 GC (10 m x 0.25 mm)	320 ºC oven temperature for sample gaseous transition	<5	Claus et al., 2006
GC	Mass Spectrometry (MS) electron impact mode	Wheat wholemeal	Sample ground, free amino acid extraction with 0.01 M HCl, and EZ-Faast kit derivatization	None	Agilent 5975 in electron impact mode with Zebron-ZB-AAA capillary column (10 m x 0.25 mm)	310, 320, 310 ºC for oven transfer line and ion source temperatures, respectively flow rate: 1.5 mL min^{-1}	6.83-21.15	Curtis et al., 2009, 2018; Muttucumaru et al., 2006
GC	MS	Ground wheat wholemeal	Sample ground, free amino acid extraction with 0.01 M HCL, and EZ-Faast kit derivatization	None	Zebron ZB-AAA column with an HP 6890 GC (10 m x 0.25 mm x 0.15mm)	320 ºC oven temperature, flow rate: 22.154 ml min^{-1}	n/a	Wilson, Guttieri, Nelson, Fritz, & Tilley, 2020
GC	Mass selective detector	Wheat flour mill streams	Sample milled, free amino acid extraction with 50 % ethanol, and EZ-Faast kit derivatization	Norvaline	Zebron ZB-AAA column (10 m x 0.25 mm)	320 ºC oven temperature, flow rate: 0.9 ml min^{-1}	n/a	Liu, Ohm, Hareland, Wiersma, & Kaiser, 2011; Ohm, Simsek, & Mergoum, 2018
Anion exchange chromatography	Pulse amperometric detector	Wheat flour	Sample milled; free amino acid extracted with water	None	AminoPac PA10 column (250 x 4mm)	Water, 250 mM sodium hydroxide, and 1 M sodium acetate eluent, flow rate: 0.25 mL min^{-1}	n/a	Li, Tyl, Kaiser, & Annor, 2019
^{1}H-NMR	600.0528 MHz spectrometer with 5 mm selective inverse probe	Ground wheat wholemeal	D_2O-CD_3OD (80:20 v/v)	d_4-TSP	Bruker Avance spectrometer	Water suppression pulse sequence with 90 º pulse and 5 s relaxation, 128 scans	3.0 – 52.6	Corol et al., 2016
^{1}H-NMR	700 MHz NMR spectrometer with 5 mm selective inverse probe	Milled whole-wheat kernel	Sample milling, free amino acids extraction with D_2O-CD_3OD (80:20 v/v)	d_4-TSP	Bruker NMR Spectrometer	Water suppression pulse sequence with 90 º pulse and 5 s relaxation, 64 scans	n/a	Navrotskyi, Baenziger, Regassa, Guttieri, & Rose, 2018
Enzymatic	UV 1800 Shimadzu spectrophotometer (UV/VIS Spec)	Ground wheat wholemeal	Whole-grain ground, deproteinized with 1 M perchloric acid and titrated with 2 M KOH. Free ASN analyzed using Megazyme kit	None	Megazyme (K-ASNAM L-Aspartate/ L-glutamate/ Ammonia) kit	n/a	8.1 %	Lecart et al., 2018

With the appearance of acrylamide-related food safety risks, a rapid, novel, and user-friendly method for free ASN analysis of wheat grown in simple laboratory environments is needed, e.g., for testing of wheat samples at ports or inland terminals. A rapid method that involves an enzymatic kit has appeared in recent years and was adopted by Lecart et al. in 2018 for free ASN quantification in wheat wholemeal samples (Lecart et al., 2018). To the best of our knowledge, a systematic comparison of the data quality of free ASN content in wheat assessed by different methods is lacking. Therefore, recommendations on the suitability of rapid testing methods for free ASN determination without a comprehensive assessment of the test's quality, effectiveness, and robustness would be premature.

As per European Commission Decision (2002), accuracy describes the closeness between the test result and a reference value, normally expressed in percent of variation (European Commission, 2002). Precision measures the closeness between replicated measurements, which is normally expressed as imprecision through the standard deviation of the results obtained under a stipulated (predetermined) condition. Sensitivity refers to the slope of a calibration curve, indicating the response of detection to a change in the concentration of the compound of interest (Bièvre & Günzler, 2005). The objective of this paper is to compare a rapid enzymatic test method with two established methods, considering these various data quality parameters.

4.3 Materials and Methods

4.3.1 Materials

Two types of materials were used for this study: L-Asparagine (ASN) with ≥ 98 % purity that was acquired from Cedarlane Corporation (Burlington, ON, Canada) and whole-wheat flour from eight western Canadian wheats grown in Manitoba, Canada.

The standard L-ASN powder was used to make two reference stock solutions (15 and 60 mg L^{-1}) for the analyses of method quality parameters, including accuracy, precision, and sensitivity, as well as the cost of time and materials needed for each sample in an experiment.

Eight western Canadian wheats consisting of six Canada Western Red Spring varieties (CDC Plentiful, AAC Brandon, AAC Cameron, BW5011, AAC Elie, Glenn), one Canada Prairie Spring Red variety (SY Rowyn), and one Canada Northern Hard Red variety (Prosper) were used in this study. These eight western Canadian wheats were grown in two locations for two years in Manitoba, Canada, in each case having 100 kg ha^{-1} nitrogen fertilizer applied. Wheats were grown on Lilyfield and Carberry in 2018, and in Lilyfield and Grosse Isle in 2019. This resulted in 32 treatments in total for free ASN analyses (Xie et al., 2021). The wheat grains from each treatment were milled into whole-wheat flour and free ASN analysis was conducted in triplicate in the laboratory for all three methods.

Harvested wheat grains were cleaned, tempered to 16.5 % moisture content over 24 hours, and milled into straight grade flour. Grains were milled to pass through a 150 µm sieve at a 75 % extraction rate using a Buhler laboratory flour mill (MLU-202) with a Buhler impact finisher (MLU-302) to produce a straight grade white flour. To prepare whole-wheat flour, the bran portion from the Buhler mill was milled with a Jacobson hammer mill to pass through a 1190 µm screen. This milled bran was added to the straight grade flour from the Buhler mill to produce whole-wheat flour samples for asparagine analysis. The moisture content of the whole-wheat flour was determined according to AACCI Method 44-15.02 prior to free ASN analysis.

4.3.2 Chemicals and reagents

4.3.2.1 UPLC-PDA method

L-asparagine and L-norvaline standards were obtained from Sigma-Aldrich (Milwaukee, WI, USA). AccQ•Tag Ultra derivatization kit and AccQ•Tag Eluents were purchased from Waters Limited (Mississauga, ON, Canada). Hydrochloric acid, acetonitrile, and filtration membranes were purchased from Fisher Scientific (Whitby, ON, Canada). Water was deionized and then filtered using a 0.2 µm Millipore filter membrane purchased from Fisher Scientific (Whitby, ON, Canada).

4.3.2.2 ^{1}H-NMR method

Deuterium oxide (D_2O), deuterated methanol (methanol-d4 or CD_3OD), TSP-d4 (sodium salt of trimethylsilylpropionic acid) and 5 mm NMR tubes were purchased from Sigma-Aldrich Canada Co (Oakville, ON, Canada).

4.3.2.3 Enzymatic method

Trichloroacetic acid (TCA) and potassium hydroxide (KOH) were purchased from Sigma-Aldrich Canada Co (Oakville, ON, Canada). The K-ASNAM L-Asparagine/L-Glutamine/Ammonia Assay Kit (Megazyme, Illinois, USA) was acquired from Cedarlane Corporation (Burlington, ON, Canada). The kit contains pH 4.9 buffer, pH 8.0 buffer, nicotinamide-adenine dinucleotide phosphate (NADPH), glutaminase suspension, glutamate dehydrogenase suspension (GIDH), asparagine suspension, ammonia standard solution, and L-Asparagine control powder. An Ultrospec 1100 Pro Ultra Violet/Visible spectrophotometer (UV/VIS) (Amersham Biosciences, Little Chalfont, UK) was used to measure the UV absorbance of the sample solutions in cuvettes.

4.3.3 Methods

4.3.3.1 Quantification of free asparagine by UPLC-PDA method

Free amino acids from whole-wheat flour (200 mg) were extracted with 0.01 M hydrochloric acid solution and were derivatized with an AccQ•Tag Ultra precolumn derivatization amino acid kit. The derivatization kit included borate buffer (which contains antioxidants and boric acid, used to adjust the pH and stabilize the solution), Aqc (a labeling reagent to attach to the amino acids through the carbamate linkage of Aqc, and thus to be fluorescent under the PDA from UPLC), and acetonitrile (used as a solvent to dissolve Aqc and the amino acids) (Boughton et al., 2011; Cohen, 2005; Weiss, 2005). UPLC analysis was conducted with an AccQ-Tag Ultra C18 column using mobile phases in a linear gradient as previously reported (Malunga et al., 2019; Xie et al., 2021). The linear gradient of mobile phases helps to separate amino acids based on their polarity.

The internal standard L-norvaline (L-NVA) with a constant concentration of 29.4 mg L^{-1} was weighed into the 0.01 M hydrochloric acid solution so that it was incorporated into each sample during extraction. Triplicate analysis was conducted to determine the response factor (rf =1.022) between free ASN and L-NVA at 260 nm, which means that at 260 nm, free ASN has 1.022 times

the peak area of L-NVA when both compounds are at the same concentration. The purpose of the internal standard was to prevent any fluctuation of recovery rate between samples affecting the accuracy of measurements.

UPLC analyses were performed using a Waters H class ultrahigh pressure liquid chromatograph, equipped with quaternary solvent manager, sample manager, heated column compartment, and photodiode array detector (Waters, Mississauga, ON, Canada) as per Malunga et al. (2019) and Xie et al. (2021). A Waters AccQ•Tag Ultra C18 column (2.1x100mm) was used for separation of extracted components.

4.3.3.2 Quantification of free asparagine by ^{1}H-NMR

The procedures described in Baker et al. (2006) and Corol et al. (2016) with some modifications were followed for ^{1}H-NMR sample preparation. From a solution of D_2O: CD_3OD (80:20) containing 0.05 % w v^{-1} TSP-d4, 1 mL was mixed thoroughly with 30 mg of whole-wheat flour in a 1.5 mL microcentrifuge tube using a vortex mixer. The mixture was heated at 50 °C in a water bath for 10 min, cooled down to room temperature (20 °C) and centrifuged at 10,000 g for 5 min. Then 800 µL of the supernatant was transferred into a 1.5 mL microcentrifuge tube and heated at 90 °C in a water bath for 2 min to stop enzyme activity. The sample was cooled at 4 °C for 45 min followed by centrifugation at 10,000 g for 5 min. From the resulting supernatant 700 µl was transferred to a 5 mm NMR tube.

^{1}H-NMR spectra of the sample were acquired at 300°K using an Avance III Spectrometer 500 (Bruker BioSpin, Coventry, UK) operating at 500.1300 MHz and equipped with a 5mm selective inverse probe. Spectra were collected using a water suppression pulse sequence with a 90° pulse and a 5 sec longitudinal relaxation delay as per Corol et al. (2016). Each spectrum was acquired using 512 scans of 65536 data points with a spectral width of 7507.507 Hz. ^{1}H chemical shifts were referenced to TSP-d4 at δ0.00. Free asparagine concentration was quantified within the δ2.9755–2.9255 region. As suggested by Topspin Users Guide (2005), the free asparagine concentration was quantified by integrating ASN peaks relative to the TSP peak of constant concentration (0.05 % w v^{-1}) in each sample so that the integration of ASN was in units of rel, meaning relative integral value (TopSpin, 2005).

4.3.3.3 Quantification of free asparagine by a rapid test (Enzymatic method)

For enzymatic quantification of free asparagine content in whole-wheat flour, the procedures described in Lecart et al. (2019) and Soares et al. (2020) were used with some modifications. Whole-wheat flour (200 mg) was mixed with 1.6 mL of 10 % w v^{-1} ice-cold TCA and stirred for 30 min on a magnetic stirrer to deproteinize the whole-wheat flour (Lecart et al., 2018; Sedgwick et al., 1991; Soares et al., 2020). The sample was then centrifuged at 4,000 g for 20 min and 1 mL of the supernatant was collected in a 10 mL glass beaker. The pH of the solution was adjusted to 8 by adding 1M KOH before placing the sample in an ice bath for 20 min, which is to achieve an optimal pH range for the enzymatic reaction and also to enhance precipitation of protein (Lecart et al., 2018). The refrigerated solution was filtered through a 0.45 µm syringe filter and 100 µL of this deproteinized sample was transferred into a 1.5 mL spectrophotometric cuvette.

A series of four main enzymatic reactions occurred after the completion of sample preparation. UV absorbance readings were taken for a blank ($A1_{blank}$, $A2_{blank}$, and $A3_{blank}$) and the deproteinized sample ($A1_{sample}$, $A2_{sample}$, and $A3_{sample}$) at the end of the first, the second, and the fourth main reaction steps. For quantification purposes, a blank that contains 100 µL of distilled water was prepared to identify the baseline of UV absorbance in each reaction. It was noted that mixing the solution in both blank and sample cuvettes thoroughly after the addition of each chemical reactant was essential for the accuracy and precision of the enzymatic method.

In the first reaction, 100 µL of the pH 4.9 buffer and 10 µL of glutaminase was added to both the blank and the deproteinized sample in the cuvette. A period of 5 minutes incubation allowed the enzymatic reaction to take place

($L - glutamine + H_2O \xrightarrow{glutaminase} L - glutamate + NH_4^+ + OH^-$) , where there was the conversion of L-glutamine into L-glutamate and ammonium ions using glutaminase enzyme (Fagan & Palfey, 2010). This reaction serves the purpose of removing glutamine interference in the whole-wheat flour samples for subsequent reactions to ensure there is accurate quantification of free asparagine concentration in the samples (Abaji & Krajinovic, 2019; Megazyme, 2018b; Nguyen, Durden, & Lavie, 2017). This reaction was followed by the addition of 150 µL of the pH 8.0 buffer together with 100 µL of NADPH and 700 µL of distilled water to both the blank and

sample cuvettes. After a second 5 minutes incubation period, the absorbance $A1_{blank}$ and $A1_{sample}$ was measured at 340 nm. The purpose of the absorbance readings was to determine the initial UV absorbance after the addition of NADPH in the cuvettes ($\Delta A1 = A1_{sample} - A1_{blank}$). At 340 nm, the NADPH has optimal absorbance.

In the second step, 10 µL of glutamate dehydrogenase (GIDH) was added to the blank and sample cuvette followed by a third 5 min incubation period to allow the reaction to complete ($NH_4^+ + 2 - oxoglutarate + NADPH \xrightarrow{GIDH} L - glutamate + NADP^+ + H_2O$). The UV absorbance was measured then for both blank and sample cuvettes ($A2_{blank}$ and $A2_{sample}$) at 340 nm. Before the addition of glutamate dehydrogenase (GIDH), the ammonium ions did not convert NADPH to $NADP^+$ (Bezkorovainy, 2002). The purpose of the second step was to determine the amount of ammonium ions that had formed through the conversion of NADPH to $NADP^+$. This conversion causes a drop in the UV absorbance reading, because $NADP^+$ has a lower absorbance value at 340 nm. $\Delta A2 = (A2_{sample} - A1_{blank})$ was used to quantify the difference between the sample and blank cuvette.

In the third step of the reaction, 10 µL of asparaginase was added to the blank and sample cuvette, followed by a final 5 min incubation period to allow the reaction to take place ($L - asparagine + H_2O \xrightarrow{asparaginase} L - aspartate + NH_4^+ + OH^-$). The purpose of the reaction was to hydrolyze L-asparagine to form L-aspartate and ammonium ions using asparaginase (Curtis et al., 2019). Due to the asparaginase enzyme specificity, asparaginase can react with both asparagine and glutamine (Abaji & Krajinovic, 2019; Nguyen et al., 2017). Therefore, it is necessary to incorporate glutaminase in the first reaction steps to remove all the free glutamine in the whole-wheat flour sample before introducing asparaginase to the system.

The generation of ammonium ions entered into the final step ($NH_4^+ + 2 - oxoglutarate + NADPH \xrightarrow{GIDH} L - glutamate + NADP^+ + H_2O$). The ammonium ions that were released from the third reaction triggered the formation of $NADP^+$ from the excess amount of NADPH and GIDH in the cuvette, causing a further drop of the UV absorbance reading. The final absorbance was

measured at 340 nm for the blank and sample cuvettes ($A3_{blank}$ and $A3_{sample}$) . The amount of L-asparagine was quantified by $\Delta A_{ASN} = \Delta A2 - \Delta A3$.

After the four steps mentioned for this enzymatic method, the weight of free ASN in the sample cuvette was then quantified using the formula: $Wt_{ASN} = \frac{V \times MW}{\varepsilon \times d \times v} \times \Delta A_{ASN}$, where V = total volume of final solution in the sample cuvette (1.18 mL); MW = molecular weight of ASN (132.12 g mol^{-1}); ε = extinction coefficient of NADPH at 340 nm (6300 mol^{-1} cm^{-1}); d = length of light path (1 cm); v = sample volume (0.1 mL).

4.3.4 Quality parameters for method comparisons

Accuracy

Two reference materials with concentrations of 15 and 60 mg L^{-1} were prepared using pure L-ASN dissolved in distilled H_2O. The two reference materials were analyzed by all three methods in triplicate (n=3) and results were compared against the known concentration to determine the accuracy of the methods. The accuracy reflects how close the measured value is to the true value as established by the International Organization for Standardization (ISO) (Bièvre & Günzler, 2005). The accuracy was calculated as: Accuracy (%) = $(\frac{measured\ value}{true\ value})$ * 100 %.

Precision

Precision was determined through measurement of the repeatability of free ASN concentration measurements in triplicate using the coefficient of variation (CV %) (Bièvre & Günzler, 2005) . The two reference materials of L-ASN standards (15 and 60 mg L^{-1}) were sub-sampled and used to determine the repeatability of all three methods in triplicate (n=3). Repeatability was calculated as: CV % = $(\frac{standard\ deviation}{mean\ value})$ * 100 %. A lower CV % value indicates better repeatability between triplicate measurements (Perini, de Oliveira, dos Santos Ornellas, & Palha de Oliveira, 2005).

Sensitivity

The sensitivity of detection instrument used for each method was measured based on the slope of the calibration curves ($slope = \frac{\Delta signal}{\Delta concentration}$) that was generated using dilution series made from

three L-ASN standard solutions. As per the International Union of Pure and Applied Chemistry (IUPAC), the slope of the calibration curve refers to the calibration sensitivity, which measures the change in the instrument signal relative to the change in concentration of the stock solution. Calibration curves that are used in analytical chemistry are generally linear and are comprised of two components, the slope of the calibration curve and the baseline of the instrument signal when running a blank solution (Harris, 2010; Skoog, Holler, & Crouch, 1961). The first L-ASN standard of 49.5 mg L^{-1} L-ASN was used to prepare a calibration curve for the UPLC method, consisting of a dilution series with four concentration levels (8.4, 17.0, 32.3, and 49.5 mg L^{-1}). The second L-ASN standard of 120 mg L^{-1} L-ASN was used to generate calibration curves for the ^{1}H-NMR method. For the ^{1}H-NMR assessment, a dilution series of seven concentration levels was prepared (6, 15, 45, 60, 75, 90, and 120 mg L^{-1}). The third L-ASN standard of 60 mg L^{-1} L-ASN standard was used to prepare a dilution series consisting of five concentration levels (0.3, 5.1, 10.2, 20.3, 25.4, and 42.4 mg L^{-1}). For the enzymatic method triplicate measurements were conducted at each concentration level. The purpose was to validate this new rapid test against the two established methods.

Cost/ analysis

Experimental cost was evaluated based on the amount of samples prepared for the analysis and the total cost for conducting the experiment, including materials, chemicals, experimental consumables, and the laboratory's charged costs of the using the instruments for the analysis. Time cost was evaluated based on the sum of time required for each of the main steps of the analysis on average, and the average number of samples that could be prepared in a day.

Correlations between methods

To evaluate the relationship between the rapid test and the two standard methods, 32 whole-wheat flour samples were analyzed by all three methods in triplicate. Relationships between the enzymatic test versus the UPLC and the ^{1}H-NMR methods were evaluated from the Pearson's correlations coefficient (r) for the linear regression of the relationship between methods.

4.3.5 Statistical analysis

JMP Pro 16 (SAS Institute Inc., Cary, NC) was used to perform statistical analysis. Free ASN concentrations for all wheat samples are presented as the mean ± standard deviation. Linear

regression was used to establish the calibration curve for all three methods. Pearson's correlation analysis was used to analyze the relationship between the enzymatic method and the two established methods.

4.4 Results

The quality parameters for the three analytical methods of free ASN quantification are shown in Table 4.2. The accuracy levels of the UPLC method for the two reference materials (15 and 60 mg L^{-1}) were close to 100% at 102.7 % and 99.8 %, respectively. The ^{1}H-NMR method at the lower concentration level (15 mg L^{-1}) had an accuracy of 126.1 %, while it was 109.4 % at the higher concentration (60 mg L^{-1}). The rapid test showed an accuracy value of 93.2 % and 93.5 % for the lower and higher concentrations, respectively. This indicated that the UPLC method provided the most accurate analysis of free ASN concentration. The accuracy level of the ^{1}H-NMR method improved when the free ASN concentration in the analyte increased. The rapid test consistently measured less free ASN concentration than the true value at both concentration levels.

In terms of the repeatability of the analysis (Table 4.2), the UPLC method had CVs of 0.58 % and 2.13 % for the lower and higher concentration levels (15 and 60 mg L^{-1}) using triplicate laboratory analysis. The ^{1}H-NMR method had 8.23 % for its CV at the lower concentration and 1.24 % at the higher concentration. The rapid test had CVs of 1.02 % and 0.44 % at the lower and higher concentrations respectively. Both UPLC and the rapid test had excellent repeatability at both concentrations of free ASN, and at the higher concentration for the ^{1}H-NMR method. However, the repeatability of the ^{1}H-NMR method was compromised at the lower free ASN concentration. The repeatability of triplicate measurement for free ASN concentration extracted from whole-wheat flour samples was measured as well. The average CV for the UPLC, the ^{1}H-NMR, and the enzymatic method was 3.2%, 9.4%, and 3.5%, respectively. The range of CV for the UPLC, the ^{1}H-NMR, and the enzymatic method was 0.4-7.7%, 1.3-13.9%, 0.8-8.8%, respectively.

The slope of the calibration curve for each method was used to determine the sensitivity parameter. The calibration curve for the UPLC method had a strong linear relationship with Pearson's correlation r = 0.9989 and the slope of the linear regression was 3679.1 AU s L mg^{-1} with y-intercept of -1077.3 AU s (Figure 4.1a). The calibration curve plot for the ^{1}H-NMR method had

Pearson's correlation r = 0.9986 and the slope of the calibration curve was 0.000059 rel L mg^{-1} with a y-intercept of 0.00064 rel (Figure 4.1b). The Pearson's correlation of the enzymatic method was r = 0.9916 and the slope of the calibration curve was 0.0389 AU L mg^{-1} with y-intercept of 0.0713 AU (Figure. 1c).

From the cost and testing time perspective (Table 4.2), the enzymatic method had the least costs associated with chemicals and the usage of UV/VIS Spec instrument and a reasonable duration of experiment (40-50 minutes per sample). The UPLC method had a slightly higher cost associated with the derivatization chemicals and UPLC instrument than the enzymatic method. But the UPLC method required a shorter duration (25-30 minutes per sample) for the experiment than the enzymatic method. The ^{1}H-NMR method had the longest experiment time (120-130 minutes per sample) and the highest cost associated with the deuterated chemicals and the NMR instrument among all three methods. As compared to the enzymatic method, the ^{1}H-NMR method had double the cost and triple the experimental time (Table 4.2).

Relationships between the free ASN concentration measured by the UPLC and by the rapid test (Figure 4.2 A), and between the ^{1}H-NMR method and the rapid test (Figure 4.2 B) were also studied using ASN extracted from whole-wheat flour samples. Pearson's correlation coefficients (r) and the linear regression formula were calculated for the linear correlations. The correlation between UPLC and the rapid test was very high with r = 0.9970 and the linear regression of the relationship had a slope of 1.028 that close to y=x, and a y-intercept of -18.896 μg g^{-1}. The correlation between the ^{1}H-NMR method and the rapid test was not as good, with r = 0.9081 and the slope and y-intercept of the linear regression being 0.828 and 65.498 μg g^{-1}, respectively.

Table 4. 2: Comparison of quality parameters for UPLC, ^{1}H-NMR and enzymatic methods for the detection and quantification of free ASN concentration using L-ASN standards.

Analytical method	Accuracy (%)		Precision (CV %)		Sensitivity	Cost /analysis	
	15 mg L^{-1}	60 mg L^{-1}	15 mg L^{-1}	60 mg L^{-1}		Experimental (CAD$)	Time (min)
UPLC	102.7±0.6	99.8±2.1	0.58	2.13	3679.1 AU s L mg^{-1}	35	25-30
^{1}H-NMR	126.1±10.4	109.4±1.4	8.23	1.24	0.000059 rel L mg^{-1}	54	120-130
Enzymatic method	93.5±1.0	93.2±0.4	1.02	0.44	0.0389 AU L mg^{-1}	24	40-50

Figure 4.1: Calibration curve generated with L-ASN standard for a. UPLC analysis with four-point dilution series; b.^{1}H-NMR analysis with seven-point dilution series; c. enzymatic method analysis with seven-point dilution series.

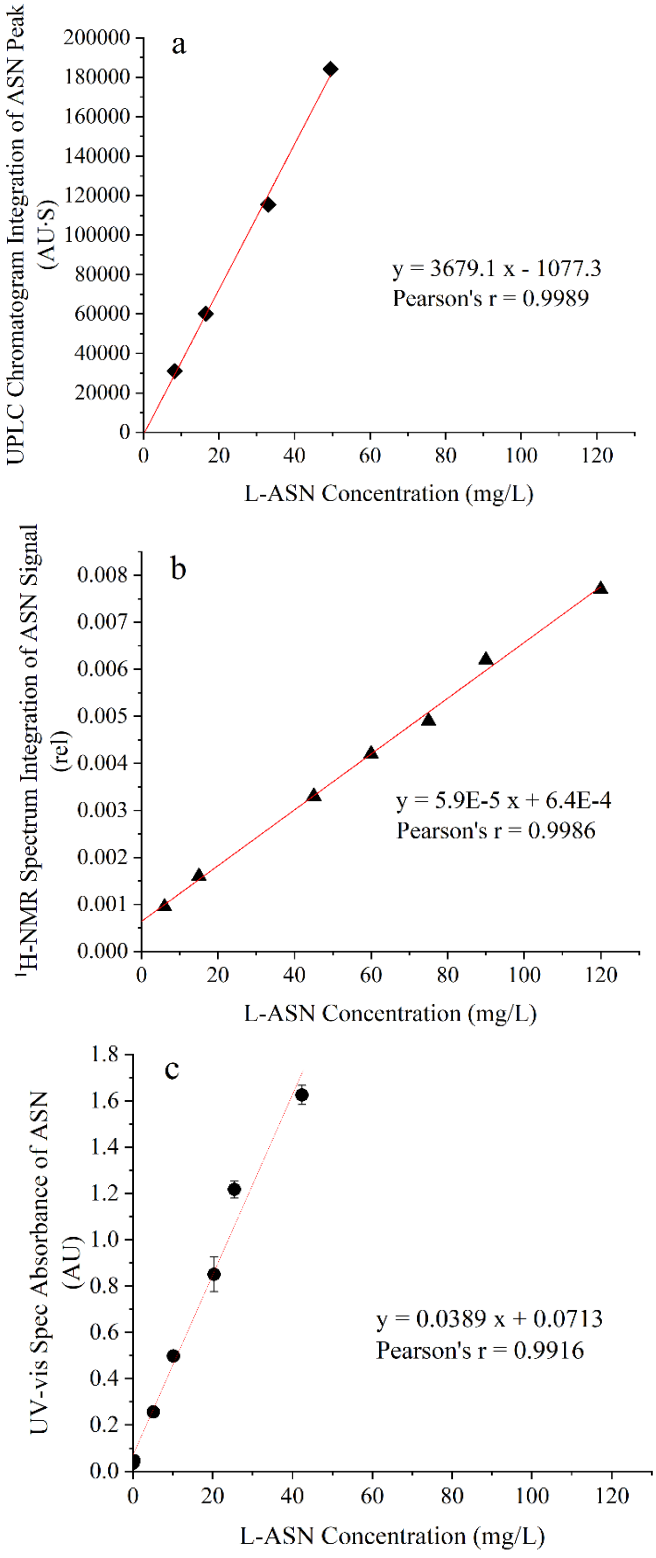
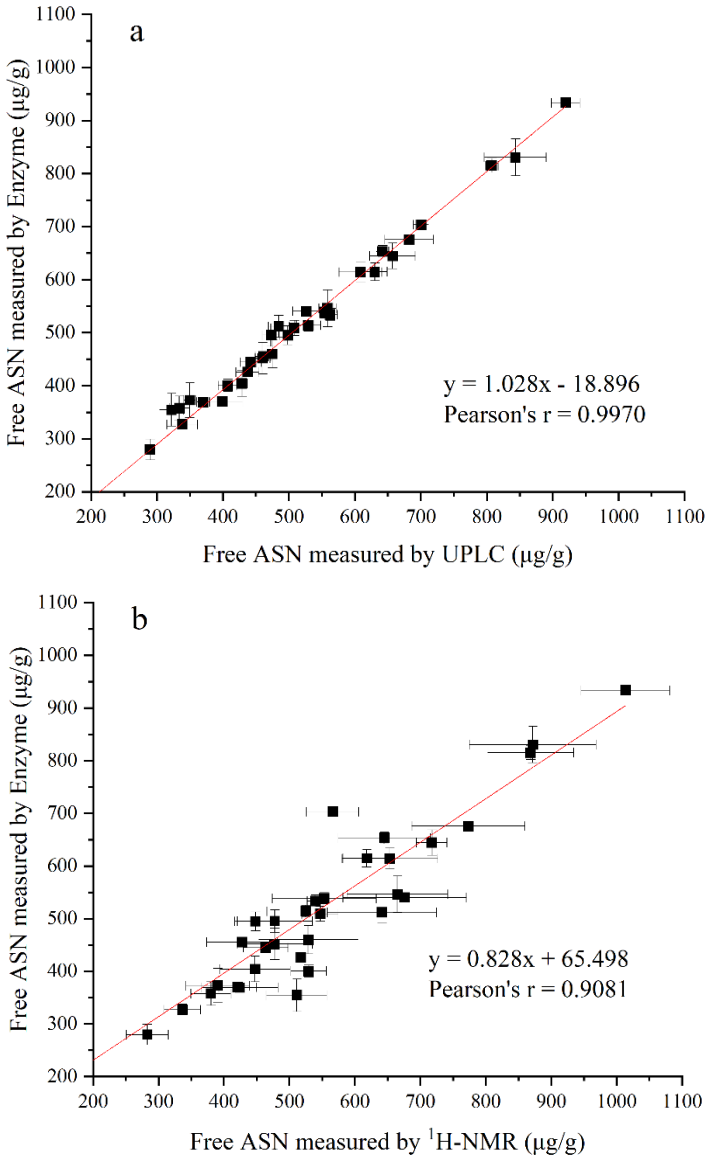


Figure 4.2: The relationship between free ASN concentration measured by a rapid test (enzymatic method) against UPLC (a), and against ^{1}H-NMR (b). The horizontal error bars represent the standard deviation of the mean free ASN concentration of each sample (n=3) measured using the UPLC (a) and ^{1}H-NMR (b), with the vertical error bars representing that measured using the rapid test.



4.5 Discussion

As described in the results section, a comprehensive comparison between the rapid test and the other two established methods is conducted. The comparison includes the performance of the three

techniques based on different quality parameters and a comparison of data for the free ASN extracted from a wide range of whole-wheat flour samples.

The accuracy of the UPLC method depends on whether the free ASN in whole-wheat flour is fully extracted using dilute hydrochloric acid solution and the quantification of the ASN peak area using the UPLC instrument. Incorporation of L-NVA as an internal standard into the free amino acid extraction step improved the accuracy of the analysis (Xie et al., 2021) by providing the ability to track all the losses occurring during the extraction of free amino acids, thereby reducing the variability of extraction efficiency between different samples and replicates (Bièvre & Günzler, 2005). Quantification of free ASN concentration through the UPLC technique was based on the ratio of the integration of the free ASN peak relative to the integration of the peak for L-NVA, which minimized variation in the extraction efficiency between samples. The use of L-NVA as an internal standard has been adopted by various research groups (Claus et al., 2006; Ohm et al., 2018). In addition, the accuracy of the flowrate, injection volume and detection affect instrument accuracy for free ASN quantification. As per the Qualification Test Specification for Waters UPLC (2016), the accuracy of the UPLC measurement is mainly influenced by variation in the injection volume. The accuracy of injection volume was >99.0 %, which means there are <1% variation in the injection volume between samples when an aliquot of the ASN sample is injected into the UPLC system. This variation of the injected sample volume will cause variations in the generation of the UPLC detection signal for both internal standard and ASN on the chromatogram (Waters Corporation, 2016). Therefore, the accuracy values obtained in this study, 102.7 % for the lower concentration standard and 99.8 % for the upper one are within expectations.

As per Liang et al. (2018), the accuracy of free ASN quantification using the ^{1}H-NMR technique was 118.5 %, which means that the quantified ASN concentration was larger than the true value. The accuracy of the ^{1}H-NMR method in this study was 109.4 % at the higher concentration standard and 126.1 % for the lower one, which partially aligned with the finding of Liang et al. (2018). Liang et al. (2018) found that the accuracy of NMR analyses ranged from 89.2% to 118.5%. The accuracy of the ^{1}H-NMR method has been reported to be influenced by instrument parameters, such as pulse sequence, relaxation delay and solvent suppression (Claridge, 2016; Hore, 2015; Liang et al., 2018). As per Claridge (1999), the accuracy of the integration on an

ordinary setting for NMR spectra may lie within 80-90 % of the true value. However, the 90º acquisition pulse that was used in this study helped to improve the accuracy by maximizing the signal intensity and increasing the signal to noise ratio. In addition, using a longitudinal relaxation time of about 5s allowed the radio frequency to recover, and to stabilize to the original state of magnetization, improving the accuracy and the recover rate of each scan (Claridge, 2016; Hore, 2015). The five second relaxation delay after a 90º acquisition pulse leads to 99.3 % recovery of magnetization, which is recommended for practical or quantification purposes (Claridge, 2016). Furthermore, the application of water suppression diminishes the signal generated by protons of the water molecules in the spectrum, which results in minimal interference of the water-induced signal with the ASN signal (Claridge, 2016). However, it is still apparent that the accuracy of the ^{1}H-NMR method in detecting a lower concentration level of target analyte is compromised. There are two possible reasons contributing to the inaccuracy of the ^{1}H-NMR method: firstly, the primary use of NMR methods is for the detection and identification of the structure of unknown compounds but not quantification of the concentration of compounds (Claridge, 2016). For quantification purposes, it is necessary to have a signal for the analyte relative to background noise in a ratio greater than a factor of 4 (Claridge, 2016). However, based on the spectrum that was acquired for preliminary experiments carried out in this study, it was challenging to maintain a ratio of 4 for the whole-wheat flour samples with low free ASN concentration. Secondly, the peak generated in the NMR spectrum by the internal standard TSP-d4 is much larger than the peak generated by ASN. This is unlike in the UPLC method, where the ratio of the peak generated by the internal standard L-NVA and the free ASN in sample are within the same scales. Therefore, quantifying free ASN concentration by using the ratio of integration between the internal standard and ASN could lead to inaccurate results.

The accuracy of the three step enzymatic reactions and the accuracy of absorbance measurements by the UV/VIS spectrophotometer contributed to the accuracy of the enzymatic method. As per Megazyme Validation Report: L-Asparagine/L-Glutamine/Ammonia Assay Kit (Rapid) (2018), the accuracy of the enzymatic method is 98.03 % (Megazyme, 2018b). In addition, the accuracy of the ultraviolet absorbance reading is within the range of 96 to 98 % (Sadegh-Zadesh et al., 2011; Wang, Wang, Xie, Zhao, & Zhang, 2019). The 2 to 4 % error is contributed by all components of the spectrophotometer, including the light source, detector, and stray light during light

transmission (Harris, 2010; Skoog et al., 1961). Therefore, 93.2 % and 93.5 % accuracy from this study (Table 4.2) falls within an acceptable range considering the errors from both enzymatic reactions and the UV/VIS Spec. For the whole-wheat flour sample, the samples with relatively high free ASN concentration are close to the upper limit of the method's working range, which would affect the accuracy of analysis. In such a situation, a preliminary analysis to identify the free ASN concentration and determine a dilution factor to bring the free ASN concentration to the optimal working range would be beneficial for the application of the enzymatic method.

In terms of the repeatability of the methods, as per Malunga et al. (2019), the precision of the ASN analysis using UPLC was within the range of 0.3 to 10.0 % (Malunga et al., 2019). The range of CV for the free ASN concentration in whole-wheat flours measured in this study was 0.4 to 7.7%, which aligned with the results generated by Malunga et al. (2019). As per Waters Qualification Test Specification for Waters UPLC (2016), the precision of the flow rate and signal detection of the UPLC instrument are <0.5 % and 2.0 %, respectively (Waters Corporation, 2016). Thus, the precision of 0.58 % and 2.13 % (CV %) for the UPLC analysis at 15 and 60 mg L^{-1} L-ASN standard solutions of this study seems reasonable after incorporating human error. Corol et al. (2016) found that the precision of the ^{1}H-NMR method for free ASN quantification in wheat flour ranged from 19.6 % to 26.7 %. As per Liang et al. (2018), the precision of ASN analysis in mulberry plant leaves using the ^{1}H-NMR technique ranged from 0.77 to 7.13 %. Therefore, the 8.23 % precision for 15 mg L^{-1} L-ASN standard and 1.124 % precision for 60 mg L^{-1} L-ASN standard and range of 1.3 to 13.9% of CV for whole-wheat flour samples of the ^{1}H-NMR method of this study are acceptable for conducting free ASN analysis in future research. The precision of the rapid test depended on the repeatability of the enzymatic reaction and the precision of the UV/VIS Spec instrument. The precision of the enzymatic reaction for L-ASN quantification validated by Megazyme was 0.93 % (Megazyme, 2018b). The UV/VIS Spec has reportedly shown acceptable precision in terms of absorbance measurement, around 0.3 %, due to its low noise to signal ratio (Currell, 2000; Skoog et al., 1961). The repeatability of the rapid test for 15 and 60 mg L^{-1} L-ASN standard solutions was 1.02 % and 0.44 %, which should be considered excellent. The repeatability of free ASN quantification extracted from whole-wheat flours was very good at a CV of 0.8 to 8.8%. Overall, for the free ASN detection and quantification, the UPLC method had the best

precision at low free ASN concentration level and the rapid test had the best precision at higher free ASN levels (Table 4.2).

In terms of the calibration curve of the three methods, the Pearson's correlation r of the UPLC, the 1H-NMR, and the enzymatic method are 0.9989, 0.9986, and 0.9916, respectively. All three methods have strong linearity, which is one of the most important factors for the development of a calibration model (Bièvre & Günzler, 2005). The strong linearity means there is a good signal response to the change in free ASN concentration in the samples. Along with the sensitivity of detection method, the limit of quantification (LOQ) is an additional important characteristics of any analytical technique (Bièvre & Günzler, 2005; European Commission, 2002).

The slope of the UPLC method calibration curve was 3679.1 AU s L mg^{-1}, which means that every 1 mg L^{-1} of increase in the ASN concentration results in 3679.1 AU s of increase in ASN peak integration on the chromatogram. The y-intercept of the UPLC method was 1077.3 AU s, indicating almost a negligible offset on the UPLC response signal when the L-ASN standard was at very low concentration. In addition, the UPLC technique had a very low limit of detection, which allows detection of free amino acids at concentrations above 6.6 μg L^{-1} at 1μL injection volume (Waters Corporation, 2007), which is equivalent to 6.6 parts per billion (ppb). The chromatography technique is one of the most popular and established method for free ASN analysis that has been adopted by many research groups due to its accuracy, precision, and sensitivity, such as Curtis et al. (2009), Çelik & Gökmen (2020), and Malunga et al. (2019 & 2021).

The sensitivity of ^{1}H-NMR in this study was 0.000059 rel L mg^{-1}, which indicated that the integration of the signal from ^{1}H-NMR spectra increased by 0.000059 rel when the ASN concentration increased by 1 mg L^{-1}. As stated by Claridge (1999), low sensitivity is a considerable drawback associated with ^{1}H-NMR comparing with other analytical techniques (Claridge, 2016). Also, a positive y-intercept was observed for the ^{1}H-NMR method, indicating the measurement of free ASN concentration tends to be consistently higher than the true values. Liang et al. (2018) found that the LOQ of the ^{1}H-NMR technique was 10.97 mg L^{-1}, which is equivalent to 11 ppm,

which is well applicable to detect free ASN concentrations encountered in white wheat flour (108 to 335 ppm) (Malunga et al., 2019).

For the enzymatic method, as per Megazyme validation report (2018), the sensitivity of the method is 0.0287 AU L mg^{-1} (Megazyme, 2018b). The sensitivity obtained by this study is 0.033 AU L mg^{-1}. A 0.0712 AU y-intercept was obtained in this study, whereas 0 AU y-intercept was obtained in the Megazyme validation report (2018). The difference between UV-vis spec instruments could result in slight variation between this experimental value and the validated value. The LOQ for the enzymatic method was 4.9 mg L^{-1}, equivalent to 4.9 ppm, which gives enough power to analyze wheat samples with low free ASN concentrations.

Working range is another critical characteristic of an analytical method (Bièvre & Günzler, 2005; European Commission, 2002). In the free ASN analysis in wheat, working range determines the feasibility of a method under various environmental, genotype, and agronomic practices across the whole world. The UPLC method can cover a substantially wide working range (Waters Corporation, 2007). The ^{1}H-NMR method covers a broader range of free ASN concentration in terms of the upper detection limit compared to the enzymatic method. The enzymatic reaction has limited range of L-ASN detection (0.424-42.4 mg L^{-1}, equivalent to 0.424 to 42.4 ppm) (Megazyme, 2018b). So, the enzymatic test may not be suitable for wheat samples with extremely high free ASN concentration, for example some of the wheat grown under sulfur deficient conditions in the UK studied by Curtis et al. (2009) and Shewry et al. (2009) with free ASN concentrations up to 8194 ppm. One possible solution to the drawback of limited working range of the rapid test method is to conduct a preliminary study to identify the range of free ASN in whole-wheat flour samples. Based on free ASN range of the sample an appropriate dilution factor can be derived so that the sample solution can be diluted to a free ASN level that fits within the working range.

In terms of experimental cost and time, the enzymatic method cost the least, while the UPLC method required the shortest analysis time. Among the three methods of this study, the ^{1}H-NMR method cost the most in terms of both experimental expenses and analysis time. In terms of simplicity (requirement for complicated analytical equipment), the enzymatic method was ranked

first followed by the UPLC method and then by the ^{1}H-NMR method. The former only requires an enzyme kit and a UV/VIS Spec for the analysis, whereas the latter relied on the presence of a highly advanced instrument for free ASN analysis. In terms of the analysis time, the UPLC method was comprised of the shortest sample preparation and data acquisition times compared to the other two methods.

In terms of the development of the linear relationship between the methods by the measurements of free ASN concentration in whole-wheat flour samples, the Pearson's correlation was r = 0.9970 and a linear regression of y = 1.028x – 18.896 was found between the UPLC method and the rapid test. The r value (0.9947) and slope (1.017) indicated a very strong correlation between the two methods. The -18.896 y-intercept indicated a tendency of the rapid test to measure less free ASN concentration than the UPLC method, which aligns with the accuracy test indicated in Table 4.2, where the rapid test measured less free ASN concentration for both reference samples. For the relationship between the ^{1}H-NMR method and the rapid test, it had a Pearson's correlation r = 0.9081 and a linear regression of y= 0.828x +65.498, indicating less correlation between these two methods as compared to the correlation between the UPLC method and the rapid test. Two possible reasons might be contributing to this lower correlation: the ^{1}H-NMR method might experience less accuracy when measuring whole-wheat flours with low free ASN concentration and, the repeatability of the ^{1}H-NMR method is not as good as the UPLC method as shown in the y-error bars from Figure 4.2. Both factors would have a negative influence on the linear correlation between the ^{1}H-NMR method and the rapid test.

4.6 Conclusions

I showed that the rapid test involving the enzymatic kit from Megazyme is able to generate reliable data for free ASN analysis based on the performance of various quality parameters by using reference materials and the measurement of free ASN in whole-wheat flour samples. Moreover, the accuracy of the rapid test was consistent throughout the whole working range. The method is also excellent in data repeatability, sensitivity of detection, and lower cost associated with experiment. In addition, unlike the other two established methods that require highly skillful laboratory personnel and sophisticated experimental instruments, the rapid test is more user-friendly so that it is feasible to be adopted in a simple laboratory environment, because it requires

only the pre-made Megazyme kit and a UV/VIS spectrophotometer. Therefore, it can be foreseen that the application of the rapid test for free ASN analysis would be a future trend.

5 General Discussion and Conclusions

In this thesis, an investigation of how the precursor of acrylamide – free asparagine (ASN), varies in western Canadian wheat was conducted. The study examined variation due to the effect of wheat genotypes (G) grown under four environmental conditions (E) that are subjected to four combinations of nitrogen and sulfur fertilization treatments (F). The measurement of free ASN in wheat was accomplished by using the advanced analytical technique of ultra-performance liquid chromatography (UPLC) with photodiode array (PDA) detection. In addition, a comprehensive study comparing the robustness, effectiveness, and quality of a new rapid enzymatic test method with two established techniques (UPLC and 1H-NMR) for free ASN analysis was conducted. Lastly, a preliminary study was performed to measure the concentration of acrylamide levels in whole-wheat bread that was made from whole-wheat flour samples varying from low to high free ASN concentrations.

The intention of understanding the acrylamide concentration in bread is because bread, as one of the most popular of foods, contributes approximately 10 to 30 % of daily acrylamide intake (JEFCA, 2005). In the study of the acrylamide formation mechanism (Figure 2.2), it was found that during the bread baking process, when the temperature is above 120 °C, the amino group from free ASN reacts with the carbonyl group of reducing sugars to form an intermediate product and then produce acrylamide (Chapter 2). In this reaction, free ASN in wheat is the primary precursor and the limiting factor that plays a critical role in acrylamide formation in bread (Muttucumaru et al., 2006a; Rapp, Schwadorf, Leiser, Würschum, & Longin, 2018; Stockmann et al., 2018).

The first key component in this thesis was to measure the concentration and understand the influencing factors of free ASN in western Canadian wheat. It was found that free ASN concentration in eight select western Canadian wheats ranged from 282 to 1014 $\mu g\ g^{-1}$ (dry basis) (Chapter 3). All three effects (G, E, and F) were significant for free ASN formation in wheat. More specifically, the growing environment, wheat genotype, and their interaction contributed over 93.7% of the variation in free ASN levels in wheat. In contrast, the effects of fertilization on free ASN concentration were only 0.7% (Chapter 3). However, in the studies done by other research groups, fertilization (especially sulfur) played a significant role in controlling free ASN concentrations in wheat grain (Granvogl et al., 2007; Muttucumaru et al., 2006b). It is critical to identify why fertilization treatment did not have a substantive influence on free ASN in western

Canadian wheat grown at the selected Manitoba locations. Regarding the effect of nitrogen on free ASN formation, in their most recent reviews, Oddy et al. (2020 & 2022) revealed that free ASN formation in wheat as a food safety threat was a consequence of wheat plants have been exposed to poor crop management, excessive nitrogen supply, or plant stress, such as salt, lack of water, and disease (Oddy, Raffan, Wilkinson, Elmore, & Halford, 2020, 2022). Under plant stress resulting from low precipitation and heat combined with excessive nitrogen supply conditions, the excess nitrogen tends to form $NH4^+$. However, $NH4^+$ is a toxic compound to the wheat plant cells, so a further plant metabolism process is induced to form free ASN from the $NH4^+$ for detoxification and to also serve as a source of nitrogen storage (Oddy et al., 2020). In this thesis, it was found that under the same fertilization condition with the same soil type, wheat grain yielded 44% more free ASN on average after exposure to higher crop heat units and lack of precipitation in Lilyfield 2019 compared to Lilyfield 2018 (Table 3.2 and 3.3). This finding aligned with the reported findings from Oddy et al. (2020 & 2022).

An insignificant effect was observed for sulfur fertilization on free ASN formation in the wheat, as no inadequate sulfur or excessive nitrogen was detected in the soils. In all four wheat growing environments, the N/S ratios in soil for all location-years ranged from 0.32 to 3.10, significantly less than 17 (Table 3.2). A hypothesis made in Chapter 3 was that the ratio between N/S ratios in soil and wheat grain was the same. So the grain N/S ratio for all location-years should be less than 17. As per Luo et al. (2000) and Weber et al. (2008), the grain is considered under fertilized with sulfur if the grain N/S ratio is greater than 17 or if the grain sulfur content is less than 0.12% (Luo, Branlard, Griffin, & McNeil, 2000; Randall et al., 1981; Weber, Koller, et al., 2008). In the study conducted in the UK (Curtis et al., 2009), it was found that under a soil sulfur deficient condition, the grain N/S ratio ranged from 24-28 for two selected wheat genotypes. After the application of sulfur fertilizer in the soil, the grain N/S ratio was decreased to 16-19. Decreasing the grain N/S ratio significantly reduced free ASN formation in the wheat grains by a factor of 12 (Curtis et al., 2009). In this thesis, only soil nitrogen and sulfur content were measured. But it would be beneficial to gain a concrete understanding of the fertilization effect on free ASN formation in wheat if grain sulfur and nitrogen content were also measured. In addition, the measurement of grain S and N content would be helpful for understanding acrylamide formation. As per Curtis et al. (2009), the addition of sulfur fertilizer to the soil led to a significant decrease of acrylamide

concentration in the baked (20 minutes at 180 °C) whole-wheat flour sample where a strong negative linear correlation, r = -0.9727, was discovered (Curtis et al., 2009). This study (Curtis et al., 2009) revealed the connection between a higher soil sulfur content, a higher grain sulfur content, and a lower wheat free ASN. This connection and the negative relationship between soil sulfur and acrylamide in baked flour imply a potential negative relationship between the soil sulfur content and acrylamide in baked bread.

The detection and accurate measurement of free ASN concentration are crucial to this thesis. With the discovery of free ASN and acrylamide-related food safety concerns, the need for a user-friendly and rapid analysis method to quantify free ASN for industrial or simple laboratory applications becomes necessary (Chapter 4). An enzymatic method is a great choice to meet these demands. However, the enzymatic method had a limited working range that only detects samples with free ASN concentrations from 0.424 to 42.4 mg L^{-1}, and can only accurately quantify free ASN above a concentration level of 4.9 mg L^{-1} (Megazyme, 2018b). In the wheat harvest from some countries, such as Germany, the free ASN concentration in grain has a significantly wider range than that in North American countries, such as the US and Canada (Granvogl et al., 2007; Ohm et al., 2017; Xie et al., 2021). As per Granvogl et al. (2007), free ASN concentrations in wheat flour that were grown in Germany had a significantly wider range than the free ASN concentration range measured in this thesis (Chapter 4). The enzymatic method's working range may not be suitable for the study in Germany (Granvogl et al., 2007), whereas the ASN concentrations in this study (290 to 920 mg kg^{-1} in Chapter 4) fit perfectly within the enzymatic method working range (Megazyme, 2018b). During sample preparation, free ASN in wheat was dissolved and extracted from aqueous solution where the initial calculation of free ASN concentration was in mg L^{-1} (Chapter 4), aligning with the unit used for the working range of the official method validation report by the enzymatic method (Megazyme, 2018b). Values below the lower limit of quantification is not a food safety concern, as the possibility of harmful effects related to acrylamide is negligible. But, in terms of acquiring accurate free ASN concentration in wheat flour samples, a preliminary analysis using a well-established technique, such as chromatography, may be needed to identify the free ASN range of the samples prior to the application of the rapid test method.

After a thorough study on the free ASN in western Canadian wheat, it is also critical to gain knowledge of acrylamide in whole-wheat bread. To discover a potential relationship between free ASN in wheat and acrylamide in bread a preliminary study for bread acrylamide quantification was conducted. Six whole-wheat breads with replicates made from the wheat varieties with the full range of free ASN concentration (282 to 1014 μg g^{-1}) were selected to conduct this preliminary study (Table 5.1). As can be seen, the presence of acrylamide in bread was negligible regardless of the free ASN concentration in the wheat as influenced by factors of G, E, and F. The concentration of acrylamide in all the selected bread samples was below the limit of detection (10 μg kg^{-1}) using the UPLC with the PDA detector technique. This value is at least 5 times less compared to the benchmark levels of acrylamide, 50 μg kg^{-1}, in wheat bread established by the Commission Regulation of the EU (European Commission, 2017). In addition, from 2007 to 2016, Canadians consumed about 0.038 kg of bread per day on average (Statista, 2022), which means that an average Canadian consumer's bread acrylamide intake level is less than 0.38 μg. Therefore, acrylamide is not a food safety concern for the bread made with western Canadian wheat flour. In addition, unlike in the European Union, Canada has no regulation on acrylamide concentration limits in commercial foods, such as bread. One reason could be the dietary intake of acrylamide from bread products for the general public in some European countries was considerably higher than in Canada. For example, in Sweden, a series of cohort studies was performed to investigate the association between acrylamide intake and cancer that involved 45,306 Swedish men from 1997 to 2005 (Larsson, Åkesson, & Wolk, 2009). It was found that the mean daily intake of acrylamide was 36.1 ± 9.6 μg and the contribution of bread products was about 11.5 μg (Larsson et al., 2009), which was about 30 times higher than the acrylamide intake of Canadian bread consumers (Statista, 2022). In addition, a US study showed that smoking cigarettes was a main contributor of acrylamide intake (ÇEBİ, 2016): the daily acrylamide absorption in hemoglobin adducts from cigarettes was found to be three to five times higher in smokers (2.4-4 μg kg^{-1}) compared to nonsmokers (0.8 μg kg^{-1}) (ÇEBİ, 2016). Taking 75 kg as a regular men's body weight, smokers absorb about 240 μg of acrylamide from smoking, which was much higher than acrylamide intake from daily dietary sources.

Table 5.1: The acrylamide concentration of whole-wheat bread made from six selected wheat genotypes with various free ASN concentrations levels from low to high.

Wheat genotype	Fertilization treatment (kg ha^{-1})	Location year	Free ASN concentration (µg g^{-1})	Acrylamide concentration (µg kg^{-1})
Glenn	135 N + 17 S	Lilyfield 2018	282	< LOD*
CDC Plentiful	100 N	Grosse Isle 2019	322	< LOD
Prosper	100 N + 17 S	Carberry 2018	640	< LOD
Rowyn	135 N	Lilyfield 2019	653	< LOD
BW5011	135 N	Lilyfield 2019	905	< LOD
AAC Elie	135 N	Carberry 2018	1014	< LOD

*LOD – Limit of detection for UPLC with PDA technique is 10 µg kg^{-1}.

Some research gaps for future work have been found after the comprehensive study about free ASN work. Compared to the environmental conditions and fertilization treatments, genotype is a factor that may be manipulated in future research. Breeding low-free ASN wheat would be a valuable solution for acrylamide-related food safety. Also, another possible solution pathway is to breed wheat plants that are able to tolerate a higher stress level associated with disease, salt, or low precipitation so that the formation of stress-induced $NH4^{+}$ and free ASN would be controlled or reduced during plant stress (Oddy et al., 2022; Raffan et al., 2020). In terms of a bread acrylamide study, mimicking the bread consumption habits of the general public and analyzing acrylamide concentration in bread subject to different toasting levels would be valuable.

In conclusion, I have shown the significance of genotype, growing environment, and fertilization treatment on free ASN formation in western Canadian wheat. Notably: 1. stress due to heat and low precipitation strongly influenced free ASN accumulation in wheat grains; 2. the free ASN concentration in different bread wheat varieties varied by almost a factor of three; 3. Exploring sulfur adequacy in the soil would give agronomists a valuable recommendation for future fertilization practice. Furthermore, the findings of the strengths and weaknesses of the new rapid enzymatic method indicated the excellent reliability of the method to potential users for purchasers of wheat who are concerned about food safety. Also, the comparison among the three methods is

meaningful for researchers for method selection. Lastly, monitoring acrylamide concentrations in bread made with Canadian wheat is still important. However, the threat to the Canadian wheat industry for securing global market access for Canadian wheat is minimal based on the preliminary acrylamide study in this thesis. Consumers should have minimal concern for their acrylamide intake from bread due to the extremely low amount of acrylamide formation in Canadian bread.

6 References

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7 Appendices

SOP: L-Asparagine Analysis with Enzymatic Reaction

Scope

This document specifies procedures for L-Asparagine quantification using Megazyme kit

Safety Precautions

- Wear a lab coat, proper gloves, and protective glassware

Chemicals and Purpose

- Trichloroacetic acid (TCA) (10% w v^{-1}) → to deproteinize whole-wheat flour sample (remove protein from the flour)
- KOH (1 M) → to adjust pH of the solution after deproteinization

Consumables

- Disposable plastic cuvettes (1 cm light path, 1.5 mL)
- 0.45 µm syringe filter
- 5 mL syringe
- Pipettor tips

Equipment and Glassware

- UV-Spectrophotometer at 340 nm
- Centrifuge
- Fume-hood
- Analytical balance
- Pipettors (2- 20 µL, 20- 200 µL, 100-1000 µL)
- Magnetic stirrer
- Magnetic stirring bar
- Stopwatch
- Glassware: beaker (10 mL), Volumetric cylinder (50 mL and 100 mL), Volumetric flask (50 mL, 100 mL and 500 mL)
- Ice bath

Reaction Principle

1. Adding glutaminase enzyme to convert glutamine into glutamate and ammonium ions

$$\mathrm{L-glutamine} + 2\mathrm{H_2O} \xrightarrow{\text{glutaminase}} \mathrm{L-glutamate} + {\mathrm{NH_4}}^+ + \mathrm{OH^-}$$

2. Adding 2-oxoglutarate and NADPH with the GIDH catalyst to react with the ammonium ion formed from reaction 1 and present in the sample to produce glutamate and $NADP^+$

$${\mathrm{NH_4}}^+ + \mathrm{2-oxoglutarate} + \mathrm{NADPH} \xrightarrow{\mathrm{GIDH}} \mathrm{L-glutamate} + \mathrm{NADP^+} + \mathrm{H_2O}$$

3. Adding asparaginase enzyme to convert free asparagine to aspartate and ammonium ion

$$\mathrm{L-asparagine} + 2\mathrm{H_2O} \xrightarrow{\text{asparaginase}} \mathrm{L-aspartate} + {\mathrm{NH_4}}^+ + \mathrm{OH^-}$$

4. Once the ammonium ion from reaction 3 enters into reaction 4, it causes a fall in absorbance, which indicates the amount of free asparagine present in the sample

$$NH_4^+ + 2-oxoglutarate + NADPH \xrightarrow{GIDH} L-glutamate + NADP^+ + H_2O$$

Standard preparation & Calibration curve

- Ammonia

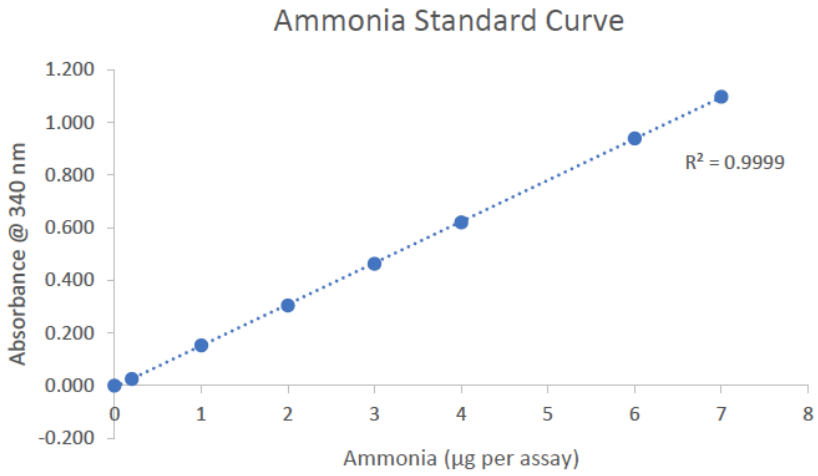
  Ammonia standard solution (5 mL, 0.04 mg mL^{-1}) in 0.02% sodium azide provided in the Megazyme kit is used.

1. Following the steps shown below, prepare the standard solutions (in the 1.5 mL spectrophotometric cuvette) and measure absorbance:

	Step 1	Step 2	Step 3	Step 4	Step 5	Step 6	Step 7	Step 8
Sample concentration µg 1.17 mL^{-1}	Stock solution µL	pH 8.0 buffer µL	NADPH µL	dH_2O µL	Incubation	GIDH µL	Incubation	ΔA ammonia
0	0	150	100	910	Incubate	10	Incubate	$(A1_{std} - A1_{blank})$
0.2	5	150	100	905	for 5 mins	10	for 5 mins	-
1	25	150	100	885	and	10	and	$(A2_{std} - A2_{blank})$
2	50	150	100	860	measure	10	measure	
3	75	150	100	835	absorbance	10	absorbance	
4	100	150	100	810	at 340 nm	10	at 340 nm	
6	150	150	100	760	(A1)	10	(A2)	
7	175	150	100	735		10		

2. Plot the calibration curve for the ammonia standard solutions (ΔA versus ammonia concentration)

Note: Example of an ammonia calibration curve (from Megazyme):



- L-asparagine

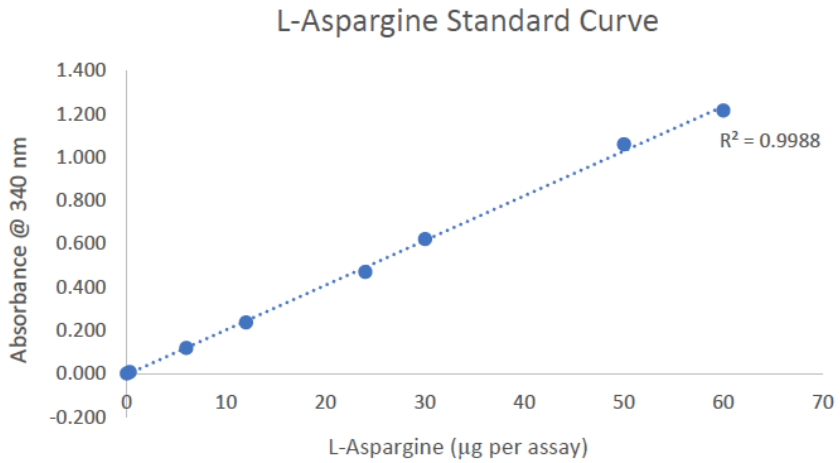
1. Weigh 0.6 g L-asparagine (provided in the kit) and dissolve in 1 L distilled H_2O to make a 600 µg mL^{-1} standard solution
2. Prepare the dilution series as follows:

Sample concentration µg 1.18 mL^{-1}	Stock solution µL	dH_2O µL	µg going into assay using 0.1 mL sample volume
0	0	1000	0
3	5	995	0.3
60	100	900	6
120	200	800	12
240	400	600	24
300	500	500	30
500	833	167	50
600	1000	0	60

3. Transfer 100 µL of the standard solutions into the 1.5 mL spectrophotometric cuvette

4. To plot the L-asparagine calibration curve (ΔA versus L-asparagine concentration), proceed with "Enzymatic reaction" steps 1-10 (page 5)

   Note: Example of an L- asparagine calibration curve (from Megazyme):



Chemical preparation procedure

Preparation of 10% w v^{-1} TCA (Under Fume hood)

1. Add 10 g of TCA (powder form with 99.0 % purity, MW: 163.39 g $mole^{-1}$) to 80 mL of distilled water
2. Mix the solution using a vortex mixer to dissolve
3. Transfer the solution into 100 mL volumetric flask
4. Add distilled water to the flask to make 100 mL of 10% (w v^{-1}) TCA solution

Preparation of 1 M KOH

1. Add 5.616 g of KOH pallet to 80 mL of distilled water
2. Mix the solution using a vortex mixer to dissolve
3. Transfer the solution into 100 mL volumetric flask
4. Add distilled water to the flask to make 100 mL of 1 M KOH solution

pH meter Calibration

Turn on pH meter, calibrate with buffer pH = 7 first, followed by calibrating with buffer with pH=4.

Sample preparation procedure

Deproteinization

1. Make an ice bath to store TCA during preparation
2. Weigh 200 mg of wheat flour sample into a 15 mL test tube and put in a magnetic stirring bar
3. Add 1.6 mL of 10% w v^{-1} ice-cold TCA and vortex
4. Stir for 30 min on a magnetic stirrer
5. Centrifuge at 4,000 g for 20 min
6. Transfer 1 mL of the supernatant in a 10 mL glass beaker
7. Add 1 M KOH (~0.545 - 0.75 mL) using 1 mL and 10 µL pipettes (make sure solution is homogenized by shaking the beaker gently) to adjust sample pH to 8 with pH probe in the beaker (Megazyme suggests maintaining pH within 7-8 range)
8. Store sample in an ice bath for 20 min to enhance precipitation
9. Filter the supernatant through a 0.45 µm syringe filter and transfer into a microcentrifuge tube (1.5 mL)
10. Transfer 100 µL of this deproteinized sample into the 1.5 mL spectrophotometric cuvette
11. Start Enzymatic reaction steps 1-10 (Page 5)

Enzymatic reaction

1. Add 100 µL of the pH 4.9 buffer and 10 µL of glutaminase to the cuvette
2. Mix solution by manually shaking the cuvette, and incubate for the 1st 5 min time with stopwatch
3. Add 150 µL of the second buffer at pH 8.0 together with 100 µL of NADPH and 700 µL of distilled water
4. Count the 2nd 5 min time with stopwatch and measure absorbance (A1) of reaction at 340 nm (converting L-glutamine into L-glutamate and ammonium ions using glutaminase). We do not need A1 for asparagine quantification, but it can be used for quantification of ammonia and L-glutamine in the samples.
5. Add 10 µL of glutamate dehydrogenase (GIDH) and mix the solution with the 100 µL pipette
6. Count the 3rd 5 min time with stopwatch and measure absorbance (A2) of reaction at 340 nm (ammonium ions are consumed by NADPH and form $NADP^+$)

7. Add 10 µL of asparaginase and mix the solution with the 100 µL pipette
8. Count the 4th 5 min time with stopwatch and measure absorbance (A3) of reaction at 340 nm (L-asparagine is hydrolyzed to L-aspartate and ammonium ions using asparaginase, absorbance of A3 drops due to the release of ammonium ions participating in reaction 2)
9. Calculate the decrease of the absorbance at 340 nm (ΔA):
   $\Delta A_{ASN} = (A2_{\text{sample}} - A2_{\text{blank}}) - (A3_{\text{sample}} - A3_{\text{blank}})$

Note: To calculate absorbance values for blank, use 100 µL of dH_2O (instead of 100 µL of deproteinized sample) in the cuvette and follow "Enzymatic reaction" steps above.

Calculation

1. The weight (g) of free asparagine in sample cuvette can be calculated as follows:

$$w_{ASN} = \frac{V \times MW}{\varepsilon \times d \times v} \times \Delta A_{ASN}$$

   where:

   - V =final volume in cuvette [mL] = 1.18
   - MW =molecular weight of asparagine [g mol^{-1}] = 132.12
   - ε =extinction coefficient of NADPH at 340 nm [L mol^{-1} cm^{-1}]= 6300
   - d =light path [cm]= 1
   - v =sample volume [mL]= 0.1

2. The mass fraction of free asparagine (g ASN g^{-1} flour) is calculated based on the dilutions carried out during sample preparation.
   For example, assume we weighed 200 mg of whole-wheat flour with moisture content of 10.284%, mixed it with 1.6 ml of 10% TCA solution and after centrifugation, we took 1 ml of the supernatant (1 mL from ~1.6 mL of mixture → dilution factor 1 (F1) = 1.6 mL 1 mL^{-1} = 1.6). Assume we had to add 0.56 mL of 1 M KOH to bring the pH of the supernatant to 8, resulting in 1.56 mL of a pH=8 solution) which was then filtered. Then we took 0.1 mL of this filtered solution (dilution factor 2 (F2) = 1.56 mL 0.1 mL^{-1} = 15.6) and used the solution for enzymatic reaction and spectroscopy and

reached to a C_{ASN} value of 0.0500 g L^{-1}. To calculate µg of free asparagine per g of whole-wheat flour sample, we have to keep track of the amount of asparagine in each step back to the beginning (when we weigh 200 mg whole-wheat flour). To do so, we follow the steps below:

- Change unit of wt_{ASN} in sample cuvette from g mL^{-1} to µg 0.1 mL^{-1}, because we used 0.1 mL of deproteinized sample for enzymatic reaction. So, we will have:

$$0.0500\frac{g}{L} = 0.0500\frac{g}{L} \times \frac{10^6\ \mu g}{1\ g} \times \frac{1\ L}{10^3\ mL} = 50.0\ \frac{\mu g}{mL} = 5.00\ \frac{\mu g}{0.1\ mL}$$

- Now, we have the amount of free asparagine (µg) in 0.1 mL of the pH 8 solution that we prepared for enzymatic reaction. To calculate the amount of free asparagine in 1.56 mL of the pH 8 solution, we have to use F2 which is 15.6. So, the amount of free asparagine in 1.56 mL of pH 8 solution will be calculated as:

$$5.00\ \frac{\mu g\ ASN}{0.1\ mL\ of\ pH\ 8\ solution} \times \frac{15.6\ \times 0.1\ mL}{1.56\ mL} = 78\ \frac{\mu g\ ASN}{1.56\ mL\ of\ pH\ 8\ solution}$$

- But this 78 µg of ASN came from that 1 mL of the deproteinized solution that we transferred from the centrifuged mixture of 200 mg whole-wheat flour and 1.6 mL of 10% TCA solution. To determine how much free asparagine we had in 1.6 mL of flour-TCA mixture, we have to consider F1 which is 1.6. Therefore:

$$78\ \frac{\mu g\ ASN}{1\ mL\ of\ deproteinized\ sample} \times \frac{1.6\ \times 1\ mL}{1.6\ mL} = 124.8\ \frac{\mu g\ ASN}{1.6\ mL\ of\ flour - TCA\ mixture}$$

- Therefore, we have 124.8 µg of asparagine in 200 mg of wheat flour (624 µg ASN g^{-1} of flour) with 10.284% moisture that we mixed with 1.6 mL of 10% TCA solution. So, the mass fraction or Dry Mass (DM) of free asparagine will be:

$$DM = 624\frac{\mu g\ ASN}{g\ flour\ basis} \times \frac{1\ g\ flour\ basis}{(1 - 0.1028) g\ dry\ basis} = 695.5\frac{\mu g\ ASN}{g\ dry\ basis}$$