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Studies on asparagine in Nebraska wheat and other grains

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STUDIES ON ASPARAGINE IN NEBRASKA WHEAT AND OTHER GRAINS

by

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STUDIES ON ASPARAGINE IN NEBRASKA WHEAT AND OTHER GRAINS

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Understanding of the contribution of environmental and genetic factors on the chemical composition of different grains is a critical issue in the area of food safety of cereal products. Numerous studies have reported that asparagine can form acrylamide, a toxic and potentially carcinogenic precursor compound, during a Milliard reaction. Therefore, studying the environmental and genetic effects that contribute to accumulation of asparagine in wheat and proso millet lines, which were grown in the state of Nebraska, is important for supporting breeding programs aimed at providing safer crops for consumers. In the realm of reduction of the asparagine concentration in wheat-based foods, another approach deals with the addition of the enzyme asparaginase from microbial sources to transform asparagine to aspartic acid and ammonia during processing. Data described herein provides, for the first time, preliminary evidence of asparaginase activity is present naturally in wheat kernels. Finally, correlation of asparagine with an unknown compound was discovered in a study of proton NMR spectrum of baked flour extract in deuterated chloroform.

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1 Chapter 1. Introduction

Since 9500 BCE, when humans first started to cultivate grains, wheat (*Triticum spp.*) has been a vital crop for the survival of ancient civilizations, and continues to play an important role in food production today. Wheat can be consumed as the main ingredient of ethnic foods (e.g., kutia) or as a raw material for the brewing industry. Mainly, however, it is used as flour for baked products. Currently, whole-wheat flour, thanks to the higher levels of dietary fibers, vitamins, minerals, and antioxidant, has become increasingly popular world-wide [1]. Aside from the numerous nutritional benefits of whole grain flour, it contains a higher concentration of free amino acids (compared to refined flour) [2]. One of these amino acids is asparagine, which, during the baking of flour, can form acrylamide [3]. In rats, acrylamide is a toxic compound with an acute oral toxicity of 107 mg/kg; a dermal toxicity of 400 mg/kg; and an LD_{50} of 124 mg/kg [4]. In humans, acrylamide is known as a “probable cancerogen” due to its neurotoxicity, carcinogenicity, and genotoxicity [5,6]. Therefore, the asparagine content in flours needs to be monitored and reduced to minimize acrylamide formation in baked goods.

It was previously reported in the literature that the level of asparagine varies among wheat cultivars, among different growing locations, and changes from year to year [7,8]. Previous literature [8] has reported the variation of asparagine in European wheats in the range of 320 – 1560 mg/kg, which was mostly driven by an environmental effect (average temperature, amount of precipitation, solar radiation, etc.). No information exists on the variation in

asparagine content in US wheats, in particular winter wheats produced in Nebraska. Therefore, in Chapter 2 of this thesis, the variation in asparagine concentration in three commercially available cultivars of hard red winter wheat (*T. aestivum*): Camelot, Goodstreak, Freeman, that were produced in five locations across the state: Lincoln (LNK), Mead (MD), Clay Center (CC), North Platte (NP), and Sidney (SYD), is reported. This information is coupled with several other quality parameters of wheat.

Chapter 3 of this thesis focuses on asparagine in proso millet, another grain important to the state of Nebraska. Proso millet is mostly cultivated for bird and livestock feed; however, proso millet is inherently gluten-free and is increasingly being used for human food and beverage production [9]. Interest in proso millet has arisen due to its ability to grow under moisture-limited conditions and has shown to increase yield when planted in rotation with hard red winter wheat [10]. Therefore, we report the variation in asparagine concentrations in different proso millet cultivars grown over 3 production years with and without irrigation.

Another efficient way to decrease the acrylamide concentration in baked products is to add an enzyme (L-asparaginase) during processing, which can reduce the concentration of asparagine. Kurman et al. [11] reported a 97% decrease in acrylamide concentration in bread crust by the addition of L-asparaginase from *Cladosporium sp.* Chapter 4 of this thesis presents preliminary data that suggests that L-asparaginase may be naturally present in the wheat kernel itself. Therefore, by studying the variation in wheat, and optimal conditions

for the activity of this enzyme, adjustment of bread making procedures could be implemented to reduce the risk of acrylamide formation and avoid the addition of exogenous L-asparaginase.

Finally, I found significant positive correlations between asparagine concentration and an unknown compound that was extracted in deuterated chloroform from wheat flour. I initially believed this compound to be acrylamide, but upon further investigation it appears to be another compound. I believe that further characterization of this compound and studying its correlation with asparagine can lead to a better understanding of acrylamide formation in during the Milliard reaction.

1.1 Justification and hypotheses

For mitigation of acrylamide development in baked products, previous research has been carried out and breeding programs were launched to reduce the level of asparagine in wheat. Halford et al. [11] reported that the level of asparagine in plants may be triggered by nitrogen and sulfur supplies; exposure to toxic metals and pathogens; and drought and salt stress. Also, due to the significant impact of environment on asparagine level, results will be specific for each growing region. Therefore, I hypothesize that collecting samples of different wheat cultivars from different parts of Nebraska state will give us an opportunity to estimate the level of asparagine from growing environments that are relevant to Nebraska (and surrounding regions) and provide recommendations to

breeders, agronomists, and producers for reducing asparagine accumulation in wheat.

A preliminary evaluation of one sample of proso millet suggested that proso millet has substantially lower asparagine compared to wheat; thus, I wanted to test this hypothesis. Also, proso millet is known as a drought tolerant crop [9]; therefore, I hypothesize that added irrigation will not have a significant impact on asparagine content in proso.

One of the most efficient ways to reduce acrylamide risk is to apply an enzyme—L-asparaginase—which can transform asparagine into aspartic acid and ammonia [12]. Usually, this enzyme is added during the dough development from bacterial sources, but preliminary data showed that L-asparaginase may be naturally present in the wheat kernel. Therefore, I hypothesize that level of asparagine can be reduced during the dough development process by L-asparaginase which is naturally present in wheat.

Finally, we wanted to apply NMR spectroscopy for measuring acrylamide concentration in baked products. NMR spectroscopy has numerous advantageous over LC-MS: does not require derivatization step and can apply for a low-cost screening of large sample sets. Therefore, NMR spectroscopy has clear advantages in screening and fingerprint application over mass spectrometric technics [13]. However, we were not able to quantify acrylamide by NMR, but we discovered the correlation between asparagine concentration and unknown compound.

1.2 Organization

This thesis is organized as follows: a literature review (Chapter 2) followed by manuscripts describing four research projects (Chapters 3, 4, 5 and 6). References can be found at the end of each chapter formatting by Chicago style.

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2 Chapter 2. Potential risks and mitigation strategies of acrylamide formation in food

2.1 Abstract

The first connection between asparagine concentration in raw ingredients and acrylamide in thermally processed foods was drawn in 2002. Since that time, this area of research has been actively developing. Significant progress has been achieved in an understanding of the mechanism of acrylamide formation due to the work of numerous research groups around the globe [1,2,3]. Study [4] helped to evaluate daily intake of acrylamide and define coffee and baked goods as major contributors of acrylamide in daily intake. Numerous research studies were focused on reducing the acrylamide concentration in final products via changing processing conditions, applying enzymes or reducing the concentration of asparagine in raw ingredients [5].

2.2 Introduction

In April 2002, the Swedish National Food Administration and a scientific group at the University of Stockholm released their findings on the presence of acrylamide in carbohydrate-rich foods [6]. Further studies showed the pathway of acrylamide formation from asparagine as a result of the Milliard reaction [1]. Since that time, the relationship between asparagine content in raw ingredients with acrylamide in thermally processed foods has evolved.

Therefore, the concentration of free asparagine in grain and factors that influence it, has drawn attention among bread makers and breeders. These studies have shown that the concentration of asparagine is mainly influenced by environment and genetic factors [7]. Corol et. al. [7] reported the variation in asparagine concentration in a range 320 – 1560 mg/kg among 150 wheat cultivars. Moreover, the same author reported a predominant influence of environment (35%) and GxE interaction (44%), meanwhile genotypic effect contributed only 13% to total variation of asparagine concentration (error= 9%) on 26 wheat cultivars from 6 environments. However, some agronomic factors such as sulfur deficient soil, presence of toxic metals in soil or pathogenic attacks can increase the concentration of asparagine in grains. Curtis et. al. [8] indicated that the level of asparagine can be reduced due to the selective breeding (from 350 mg/kg to 220 mg/kg). However the same author indicates that selected genotypes should also be tested under a range of environmental conditions.

2.3 Mechanisms of acrylamide formation

Asparagine contains an α-amino group and amide group (Fig. 1), has isoelectric point of 5.41, a molecular mass of 132.12, and pKa values 2.02 and 8.8. It is partially soluble in water and highly soluble in alkaline or acidic solution. In living organisms, due to the high N:C (2:4) ratio, it is an efficient molecule for nitrogen storage and transportation [9].

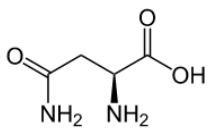

Figure 1 – Structure of asparagine

A high level of concern rose by numerous studies [1,2] that connected the concentration of the free amino acid asparagine in flour with acrylamide in a baked product. These studies [3] proposed a mechanism of acrylamide formation during the Maillard reaction (Fig. 2). It starts from the reaction between an amino group of asparagine and carbonyl group of reducing sugars, with the formation of Schiff-base intermediates. After the decarboxylation of the Schiff base, acrylamide can form by two separate pathways.

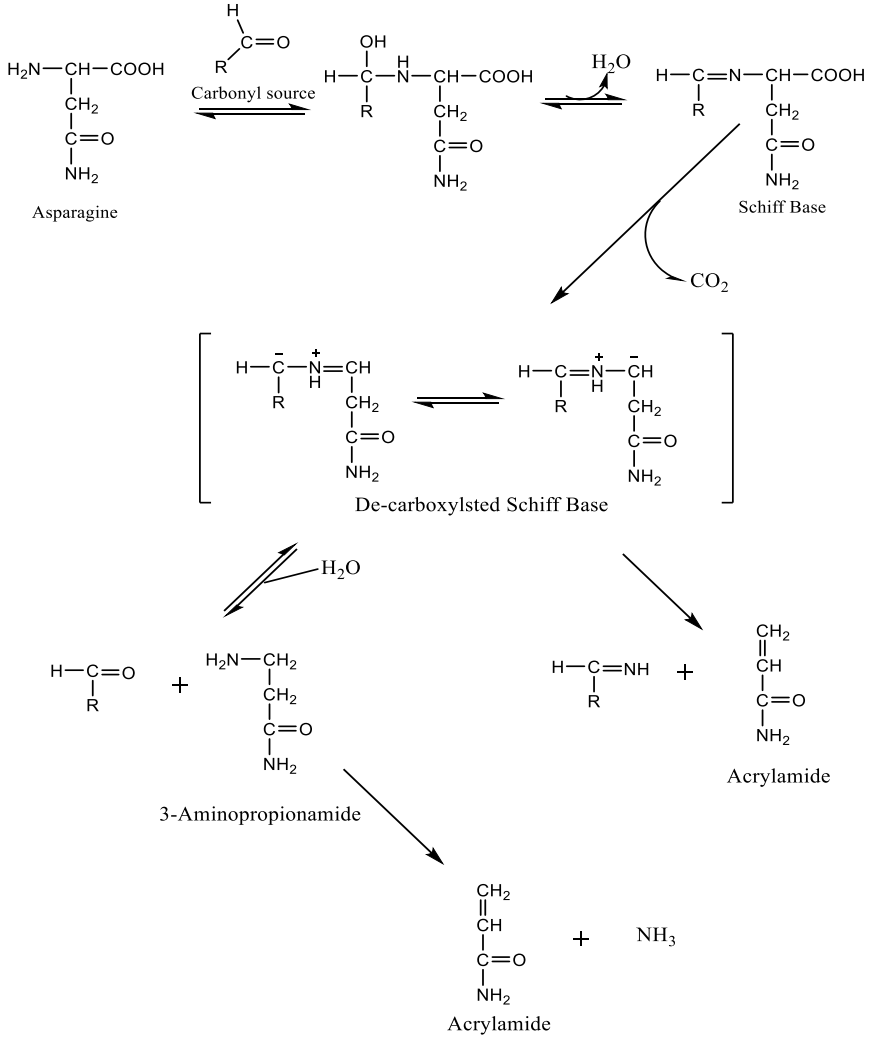

Figure 2- Mechanism of acrylamide formation [1]

2.4 Factors which contribute to asparagine accumulation in grains

Asparagine accumulates under conditions of stress as a biological response to the maintenance of osmotic pressure or due to the restriction of protein synthesis. Such stress conditions can be caused by drought, mineral deficiency, presence of toxic metals in soil and pathogenic attacks [9].

It is well known that the protein profile in wheat changes during growth in drought conditions [10]. It has been reported that proline and asparagine concentration can be significantly increased during drought or salt stress [11]. Access to minerals plays an essential role in plant development and protein formation. Deficiencies of potassium, sulfur, or phosphorus also can significantly increase asparagine concentration in plants [9]. Deficiency of sulfur, in particular, decrease the formation of cysteine and methionine, which contribute to protein aggregation by the formation of disulfide bonds. Lack of ability to efficiently bind protein leads to increase of the concentration of free amino acids [9].

It is well known that asparagine in plants is used as a nitrogen storing amino acid, due to its high N:C ratio (2:4) [9]. do Amarante et. al. [12] reported that four days of maintaining the plants in nitrogen-free conditions caused a reduction in the proportion of asparagine and an increase in aspartate in all the plants tested.

In plants, asparagine can bind to toxic metals such as cadmium, lead, and zinc by forming intracellular complexes and by these means decrease the toxicity of these metals. Therefore plants can use asparagine as the first line of defense

against toxic metals, and therefore increase accumulation of this amino acid in the condition of toxic metals threat. For example, cadmium induced almost a 10-fold increase in the concentration of asparagine in tomato roots [13].

Finally, a significant difference was observed in asparagine concentration among tomato plants that were grown in sterile conditions compared to those infected with the bacteria *Pseudomonas syringae.* Perez-Garcıa et al. [14] reported an increase of almost 1.5 fold in a group of infected plants. Another study [15] showed that infection of cocoa by *Crinipellis perniciosa* fungus can increase the concentration of asparagine significantly.

2.5 Occurrence and Dietary Intake

Acrylamide occurs in food products during baking, frying and toasting at temperatures above 180 C. The average intake of acrylamide is estimated at 0.32 ±0.19 µg acrylamide per kg body weight per day [4], with most of the acrylamide coming from coffee. However bakery products were the second significant contributor to acrylamide daily intake after coffee.

Acrylamide is classified by the International Agency for Research on Cancer (Lyon, 1994) as “Group 2A” (probably carcinogenic to humans), due to its neurotoxicity, carcinogenicity, and genotoxicity [16,17]. These decisions were motivated by studies that indicated that multi-organ tumors were discovered in experimental animals after the exposure to acrylamide [18]. A more recent study [19] showed that acrylamide has a mutagenic effects on humans because it can be converted to glycidamide (Fig. 3), which is three times higher in mutability

compared to acrylamide and can lead to mutations in various systems. In addition, acrylamide can act as a Michael acceptor to form adducts with different functional groups in DNA (amino, hydroxyl, and thiol) which cause damage to DNA [20]. Finally, exposure to acrylamide has the cumulative and chronic effect, which leads to damage of both peripheral and central nervous systems [21].

Figure 3 - Scheme of glycidamide formation from acrylamide

FDA's best advice for acrylamide and eating is that consumers adopt a healthy eating plan, consistent with the Dietary Guidelines for Americans, that emphasizes fruits, vegetables, whole grains, and fat-free or low-fat milk and milk products; including lean meats, poultry, fish, beans, eggs, and nuts; and limits saturated fats, *trans* fats, cholesterol, salt (sodium) and added sugars. FDA is waiting for new research results before considering whether new advice on acrylamide is needed [22].

2.6 Mitigation strategies for Acrylamide consumption

For providing a safe product to consumers, numerous strategies have been suggested to mitigate acrylamide formation in foods. To ensure that acrylamide will not form by the asparagine pathway, asparagine could to be reduced. Therefore the level of asparagine could be a new target that wheat

breeders could take into consideration when advancing new lines in their breeding programs.

Controlling the Milliard reaction pathway is another strategy for decreasing acrylamide accumulation in thermally processed foods. Variation of time and temperature during food processing can be crucial for acrylamide accumulation. Moreover, changing pH, moisture content of raw materials, and enzyme addition can significantly reduce the level of acrylamide in the final product.

2.6.1 Breeding of low asparagine wheat cultivars

Performing high throughput analytical screening among different wheat lines and environment conditions for monitoring the asparagine level is crucially important for supporting breeding programs aimed at reducing the potential risk of acrylamide formation in baked products. Research performed in Europe on 150 wheat samples from different countries (Hungary, United Kingdom, and France) showed that a variation in asparagine between 320-1500 mg/kg [7]. This study reported significant effects of growing environments and genetic cultivars as well as the interaction between these two factors. Also, this article proposed a new method for asparagine quantification in wheat flour by extraction in deuterated water with further NMR detection.

This study [7] also showed that the level of asparagine significantly correlated with protein and starch concentration, Zeleny sedimentation and Mixograph water absorption. In addition, asparagine concentration was correlated positively with plant height.

2.6.2 Fermentation

It is well known that L-asparaginase is found in numerous microorganisms with different activities: *Aeromonas hydrophila* (40-150 units/gram of cells), *Erwinia aroideae* (55-770), *Escherichia coli* (0-225), *Proteus americanus* (160), *Pseudomanas acidovorans* (175-210) [23]. This enzyme catalyzes the hydrolytic cleavage of the amino acid L-asparagine into L-aspartic acid and ammonia (Fig. 4).

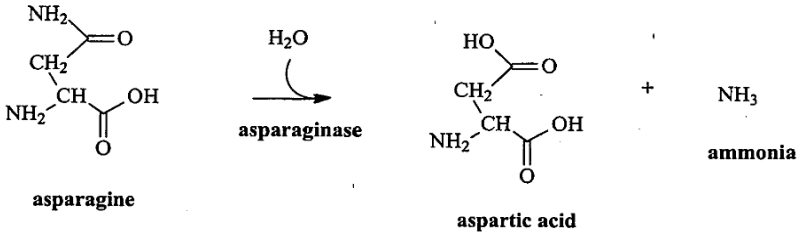

Figure 4 - Scheme of *L*-asparaginase activity

Therefore, one of the most efficient methods of reducing acrylamide formation is the addition of L-asparaginase to dough during the mixing stage. Previous research [24] has shown that L-asparaginase has the ability to reduce the formation of acrylamide in the range of 23-75 % depending on the pH of dough and time of enzyme incubation.

Therefore, more research is needed to evaluate potential presence of an *L*-asparaginase in wheat and other grains. Precise evaluation of activities of this enzyme can help to modify baking conditions to eliminate risk of acrylamide formation.

2.6.3 Other methods for acrylamide mitigation

Because of the high risk of acrylamide formation, different methods are amplified for preventing acrylamide formation during the food processing. A recent article [25] showed that addition of low molecular weight (50–190 kDa) chitosan can be used to mitigate the formation of acrylamide. An effect of chitosan can be explained by a reaction between the chitosan amino group and the carbonyl group of the reducing sugars in the Maillard reaction. Another approach is to control the baking time and temperature. Surdyk et al. [26] reported a decrease in acrylamide concentration from 2000 mg/kg to 300 mg/kg when the baking temperature of wheat bread changed from 290° C to 220° C.

2.7 Asparagine level in gluten free grains

Due to high public attention to Celiac disease and the emerging gluten free market, production of gluten-free grains is growing by 20% annually [27]. Due to the growth in utilization of gluten free flours to produce baked products, we believe that it is important to investigate potential risks of acrylamide formation in gluten free products. There are very few studies on genetics and environmental effect on asparagine level in gluten-free grains. Therefore, this area of research needs further development.

It was reported [28] that acrylamide potential of rye flour has been higher compared to wheat, due to the presence of higher level of asparagine and sugars in rye (). Curtis et al. [8] reported the variation of asparagine level in 8 varieties of rye within a range 500 -1000 mg/kg. Another study [29] investigated acrylamide

precursor contents in various amaranth and quinoa cultivars. The results indicated significant differences in the potential for acrylamide formation of quinoa cultivars and slight differences between the tested amaranth cultivars and genotypes. The results suggest that use of cultivars with low levels of free asparagine might be a feasible strategy to lower the risk of acrylamide consumption.

2.8 Conclusion

The area of acrylamide risk in foods is currently under deep concern, and more research is required for protecting consumers from exposure to acrylamide. Study of an environmental and genetics effects on asparagine level in wheat is crucially important for determine the risks of acrylamide accumulation in baked products. The concentration of asparagine in flours from other grains should also be evaluated for level of asparagine in order to provide safe products to the market. More research is needed in the area of reducing asparagine level by asparaginase which could be naturally present in wheat flour.

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3 Chapter 3. Effect of environment and genetics on asparagine and alanine concentration, total free amino acids and other characteristics of Nebraska wheat

3.1 Abstract

Because asparagine concentration in wheat depends on genetics and environmental factors, a study of different wheat varieties and growing locations is needed to know relative importance these factors and their interaction for the proper evaluation of potential risks of acrylamide formation in baked products made from Nebraska wheats. Three wheat cultivars (Goodstreak, Camelot, Freeman) along with some experimental lines (NE14434, NE14607, NE14674, NE 14606, NE14656, NE 14658, NE14696) were grown at five locations in 2014 using an augmented design and twelve locations in 2016 using an alpha lattice randomized complete block design. Results showed variation in asparagine concentration within a range of 200-1100 mg/kg. In 2014, a higher concentration of asparagine was found in North Platte, a location with the highest solar radiation among locations and possible sulfur deficiency. Correlations between asparagine concentration and kernel diameter, hardness and weight, protein level, total free amino acid concentration, yield, and other variables revealed significant positive correlations with kernel size ($r=0.367$; $p=0.004$) and weight ($r= 0.374$; $p=0.032$) which was not previously reported in literature.

3.2 Introduction

In April 2002, the Swedish National Food Administration and a scientific group at the University of Stockholm released their findings on the presence of acrylamide in carbohydrate-rich foods [1]. Stadler et al. [2] reported that acrylamide can be formed from asparagine during the Milliard reaction. Since that time, the relationship between asparagine content in unprocessed foods with acrylamide in thermally processed foods has been actively developing.

Because of the risk of exposure of the consumers to the high amount of acrylamide in daily intake numerous mitigation strategies have been applied: low asparagine breeding programs in grains; changing of food processing conditions; and enzyme addition.

Therefore, performing high throughput analytical screening among different wheat lines and environment conditions for monitoring asparagine level is crucially important to support programs aimed at reducing the potential risk of acrylamide formation in the baked products. Research performed in Europe on 150 wheat samples from different countries (Hungary, United Kingdom, and France) showed that the variation in asparagine was between 320-1500 mg/kg [3]. This study reported significant effects of growing environments and cultivars as well as the interaction between these two factors. Also, this article proposed a new method for asparagine quantification in wheat flour by extraction in deuterated water with further NMR detection, which we applied in our study.

The first study of NMR spectrum profile of wheat extract was carried out by Ward et al. [4] and appearance of amino and organic acids region,

carbohydrate and the aromatic region were introduced. In the more recent study [3] ^{1}H-NMR spectrum described above was used for quantification of asparagine. From Fig.5 we can see that asparagine signals of protons from C-3, appears in the region of 2.8-3.0 ppm and did not overlap with other signals from the extract. Results on the determination of asparagine by NMR spectroscopy reported by Coral et al. [3] showed good agreement with studies which utilized GC-MS [5,6].

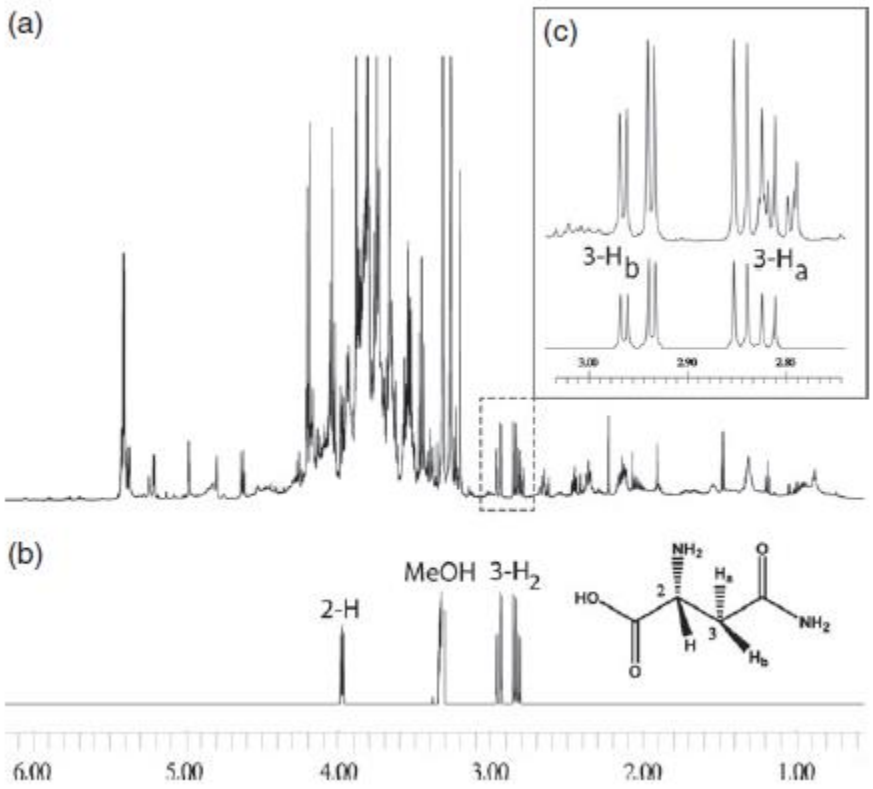

Figure 5 - NMR quantitation of asparagine in what flour. (a) NMR spectrum of typical wheat flour extract made in deuterated methanol and deuterated water (1:4). (b) spectrum of pure asparagine made in deuterated methanol and deuterated water (1:4). (c) expansion of the 3-H2 signal (top) and illustration of 3-Hb of asparagine standard (bottom) which was utilized for quantitation [3]

In this study [3], asparagine concentration significantly correlated with protein and starch concentration, Zeleny sedimentation, and Mixograph water

absorption. In addition, asparagine concentration was correlated positively with plant height.

3.3 Materials and methods

3.3.1 Statistical analysis

For analysis of G*E interaction in our study we applied augmented experimental design with 5 replications of check cultivars and 1 replication of experimental lines. Significant difference between experimental lines was calculated based on variance from check lines by using Fisher's LSD procedure in SAS. Partial correlations were drawn based on LSMeans.

3.3.2 Meteorology data analysis

Meteorology data was collected from the Automated Weather Data Network (AWDN) of the High Plains Regional Climate Center (HPRCC) for each location. The flowering dates were estimated based on growing degree days [7]. Weather variables were calculated and compared from flowering to harvest, since this period is the most important to kernel development.

3.3.3 Samples utilized in the study

For determination of the environmental and genetics effect on asparagine level and study its correlations with other parameters in hard red winter wheat from 2014 harvest year, a set of three genotypes and 5 locations were collected and complimented by the samples from the wheat breeding program (Table 1).

Table 1.Sample set from 2014

Location/ Cultivar	Camelot	Goodstreak	Freeman	NE14434	NE14607	NE14674	NE14606	NE14656	NE14658	NE14696
Lincoln	5*	5	5	1	1	1				
Mead	5	5	5	1	1	1				
Clay Center	5	5	5	1	1	1				
North Platte	5	6	5	1	1	1				
Sidney	4	4	3	1	1	1	1	1	1	1

*Values in the table represent the number of replications.

Because of the huge environmental impact on asparagine concentration in wheat samples from 2014, twelve locations were sampled in 2016 (Table 2).

Table 2. Sample set from 2016

Location/ Cultivar	Mead	Sidney	Clay Center	North Platte	Lincoln	Saunders	Lancaster	Saline	Cheyenne	Box Butte	Deuel	Kimball
Camelot	3					2	5	5	2	3	2	2
Goodstreak	2	1	1	1	2				2	3	2	2
Freeman	5	1		1	2	2	5	5	2	3	2	2
NE 14434	2	1	1	1	2							
NE 14674	2	1	1	1	2							
NE 14696	2	1	1	1	2							
NE 14606	1	1	1	1	2							

*Values in the table represent the number of replications.

3.3.4 Procedure for asparagine and alanine quantification in wheat flour

Extraction of asparagine and alanine from wheat flour was carried out according to the protocol for standard metabolomics analysis by NMR spectroscopy [8] with some modification. One mL of D_2O-CH_3OD (80:20 v/v) was added to 30 mg of flour, which was placed into 2 ml tube. The sample was then vortex mixed for 5 s and placed into a hot water bath (90° C) for 10 min. Tubes were secured horizontally in the water bath and shaken at 150 rpm. The high-temperature extraction was incorporated to ensure by denaturization that potential L-asparaginase activity would not bias the results of the analysis (see chapter 5).

After the extraction, samples were centrifuged at 5000 rpm for 10 min at 4° C. The supernatant was then transferred to another tube and placed at 4° C for 45 min. This step was necessary to help purify the sample by precipitating out less soluble material at the low temperature. The sample was then centrifuged again, and 400 µL of the supernatant was transferred to a 5-mm NMR tube. An internal standard 2,2,3,3-d(4)-3-(trimethylsilyl)propionic acid sodium salt (d4-TSP) was added to NMR tube for identification and quantification purposes.

^{1}H-NMR spectra were acquired under automation at 37° C using Bruker NMR Spectrometer operating at 700 MHz and equipped with a 5 mm selective inverse probe. Spectra were collected using a water suppression pulse sequence with a 90° pulse and a relaxation delay of 5 s. Each spectrum was acquired using 64 scans of 64 000 data points with a spectral width of 7309.99 Hz. Spectra were automatically Fourier transformed using an exponential window with a line

broadening value of 0.5 Hz. Phasing and baseline correction were carried out by the instrument software. ^{1}H chemical shifts were referenced to d4-TSP at δ=0.00.

3.3.5 Protein fractionation and quantification

Samples were analyzed using 10 mg of flour weighed out in duplicate as reported previously [9]. The first replication was treated with 1.8 ml of 50% (v/v) propan-1-ol for 30 min in a water bath at 25 °C. Each sample was vortexed for 5 s before and after the extraction. Samples were then centrifuged at 13500 x g for 5 min. Following centrifugation, the absorbance of the supernatant was measured at 280 nm and blanked with 50% (v/v) propan-1-ol. This fraction was referred to as the low molecular weight (LMW) gluten proteins (monomeric proteins or gliadin). The second tube was extracted with 1.8 ml of 50% (v/v) propan-1-ol containing 0.2% (w/v) dithiothreitol (DTT) at 55 °C. The spectrophotometer was blanked with 50% (v/v) propan-1-ol and 0.2% (w/v) DTT solution. This fraction represented the total extractable protein. The portion of high molecular weight (HMW) gluten proteins (polymeric proteins or glutenins) was then calculated as the difference between total extractable protein and LMW protein fractions. Based on data obtained by this analysis, the protein ratio (PR) was calculated by dividing the percentage of HMW by LMW proteins.

3.3.6 Moisture content

Moisture was quantified using AACC International Method 44-15.02. A duplicate of 500 mg of flour was placed into an oven at 130° C for 1 h. After cooling to room temperature in the desiccator, moisture content was calculated as the percentage of weight loss. Duplicate determinations were checked within

0.2% moisture; otherwise, the determination was repeated. All measurements were then reported on dry weight basis.

3.3.7 Reducing sugars

Reducing sugars were extracted with 50% aqueous ethanol for 30 min at 50 °C. Thirty mg of flour were dispersed in 1 ml of extracting solution. Determination of reducing sugars followed the dinitrosalicylic acid method [10].

3.3.8 Total free amino acids

Free amino acids were extracted, with 0.001 N of hydrochloric acid at room temperature for 30 min. Free amino acids were then measured by UV spectroscopy as described [11].

3.3.9 Hardness, size and weight of kernels evaluation by single kernel characterization system

Data on hardness, size, and weight of kernels was collected using SKCS 4100 (Perten, Sweden) following the manufacturer's instructions.

3.4 Results and discussion

Results from ANOVA on each response variable are shown in Table 3.

Table 3. ANOVA table from samples 2014[A]

Source of variance	Location (Df)	Cultivar (Df)	Cultivar x Location(DF)	Error df	Error
Asparagine	38873.8*(4)	36112.6*(2)	3009.4(14)	121	3102.5
Alanine	10546.3*	102.9	771.7	121	1028.9
Free amino acids	24.1*	5.6	17.3*	121	6.7

Reducing Sugars	5.7*	27.4*	10.0*	121	2.4
Protein	1.27*	4.81*	0.8*	121	0.246
Low molecular weight proteins	377.1*	1973.9*	87.2	121	50.4
High molecular weight proteins	377.1*	1973.9*	87.2	121	50.4
Protein Ratio(HMW/LMW)	0.6*	2.4*	0.2*	121	0.0661
Kernel Size	0.011*	0.269*	0.0134*	121	0.00202
Kernel Weight	12.7*	154.8*	10.9*	121	1.14
Hardness	300.7*	1016.0*	23.7*	121	8.288
Yield	10018433*	25635	316936	121	233041

[A]Values in table represent mean squares; * - designates significant differences at p≤0.05.

3.4.1 Total free amino acids

For total free amino acids, the G x E interaction was significant as well as environment effect, however, cultivar effect was not significant indicating there was little genetic variation for this trait; but the genotypes do respond differently to the environment. Total free amino acids varied from 20 mmol/ kg to 40 mmol/kg and most of the cultivars tended to have a higher concentration of free amino acids in North Platte (Fig. 6).

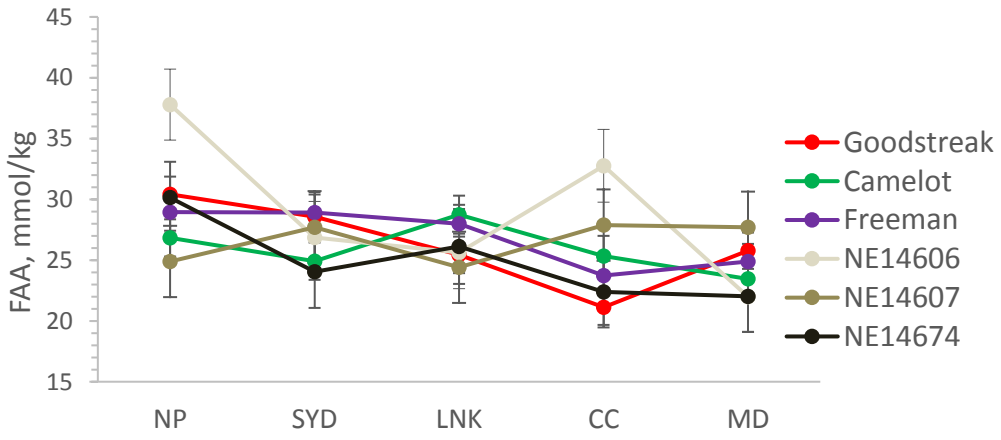

Figure 6 - Variation of free amino acid concentration among locations and cultivars

3.4.2 Asparagine and Alanine concentration

Data obtained on NMR spectroscopy employed in our study confirmed the presence of previously described asparagine peaks used for quantification at δ=2.9-2.95 (Fig. 7). I also noticed the presence of a doublet in the region of δ=1.44-1.46, which may give an opportunity to quantify another amino acid. Literature [12] suggested that this amino acid could be alanine.

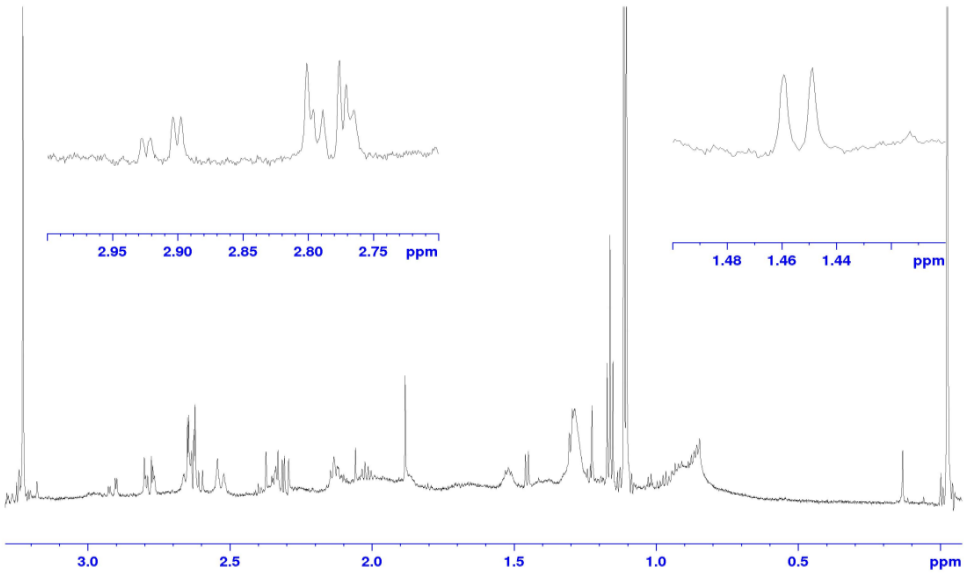

Figure 7 - NMR spectrum of a wheat extract obtained in our study

Measuring of asparagine in the sample set from 2014 was our primary outcome. Our results from 2014 showed that there were a significant effect of location as well as cultivar, with no interaction between them (Table 3). Therefore, we can interpret main effects. Samples from North Platte had significantly higher concentration of asparagine compared with other locations (Fig. 8).

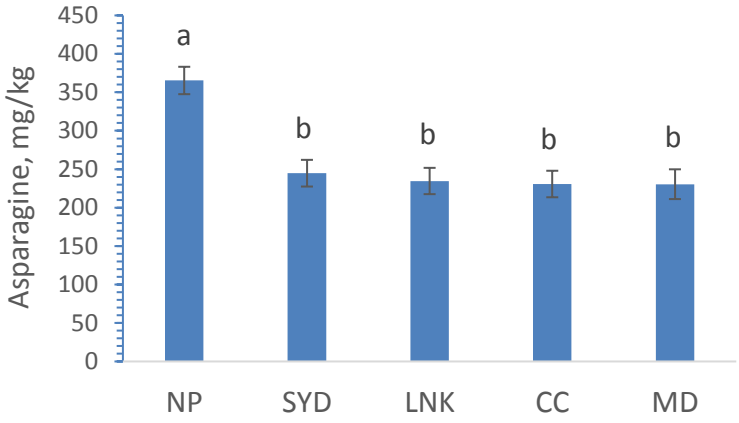

Figure 8 - Variation of asparagine concentration among the locations

Among the genotypes studied, Goodstreak had the highest concentration of asparagine followed by Camelot and NE14607 (Fig. 9). Freeman, NE14434, and NE14674, had the lowest values of asparagine.

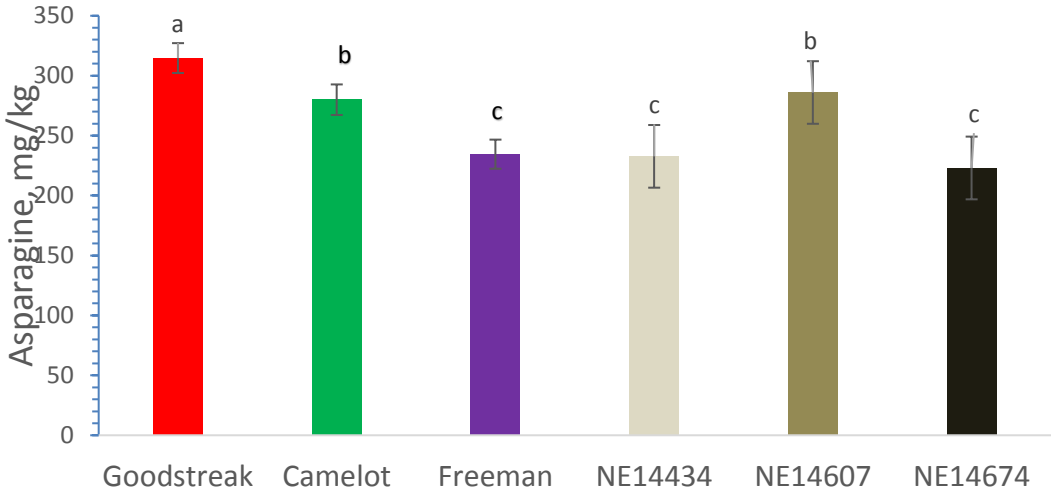

Figure 9 - Variation of asparagine concentration among the cultivars

In the case of alanine, no significant interaction between cultivar and environment was found, and the cultivar effect itself was not significant (Table 3). However, one location showed significant impact on alanine (Fig. 10). As with

asparagine, North Platte had the highest level of alanine.

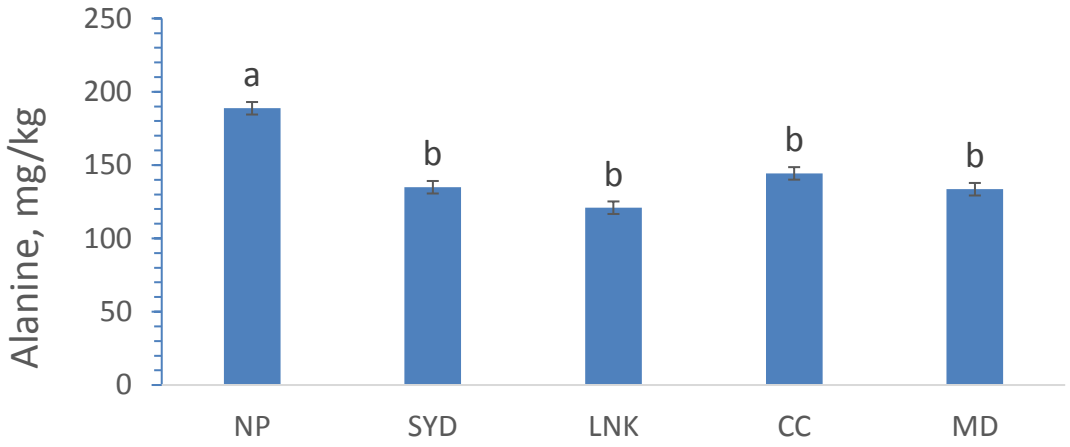

Figure 10 - Effect of location on alanine concentration in sample set from 2014

Increase in amount of asparagine (and alanine) in grains may be caused by sulfur-deficient soil [13]. Available data on soil composition across Nebraska suggested that North Platte may be in a sulfur deficient region [14].

Alternatively, North Platte had the longest period from flowering to harvest (Table 4). It was previously reported that drought stress can contribute to increased concentration of asparagine in other grains [15]. Therefore, exposure to higher solar radiation and total evapotranspiration from the surface in North Platte compared to the other locations (1002.2 MJ/m^2 and 277.76 mm) may contribute to asparagine accumulation in the seed.

Table 4. Meteorology data from 2014.[A]

Cultivar/trial	Flowering date	Days to harvest	Temp (°C, avg)	Rh (%, avg)	Soil Temp (°C, avg)	Precipitation* (mm/d, avg)	Precipitation* (mm, total)	Solar Rad (MJ/m^2, avg)	Solar Rad (MJ/m^2, total)	ET (mm/d, avg)	ET (mm, total)
North Platte	7- Jun	45	20.6	67.2	23.37	2.99	134.87	22.27	1002.2	6.17	277.8
Lincoln	12-Jun	26	22.7	71	24.36	3.44	89.41	18.86	490.51	5.85	152.1
Mead	9-Jun	33	21.8	74.6	23.38	3.64	120.14	19.64	648.22	5.45	180.1
Clay Center	9-Jun	38	21.6	74.2	23.64	3.65	138.69	23.3	885.31	6.46	245.4
Sidney	22-Jun	32	21.4	57.6	24.63	1.73	46.85	24.9	797	7.88	252.3

[A]Temp, temperature; avg, average; Rh, relative humidity; Rad, radiation; ET, evapotranspiration.
*Data on precipitation relates only to natural precipitation and do not include artificial irrigation.

3.4.3 Reducing sugars

Because reducing sugars also contribute to acrylamide formation and total free amino acids level can provide a better understanding of asparagine accumulation these parameters were evaluated in our study.

A significant G x E interaction was found for reducing sugars concentration (Table 3). Goodstreak consistently had high levels of reducing sugars among cultivars, and it reached its highest value in North Platte (Fig. 11).

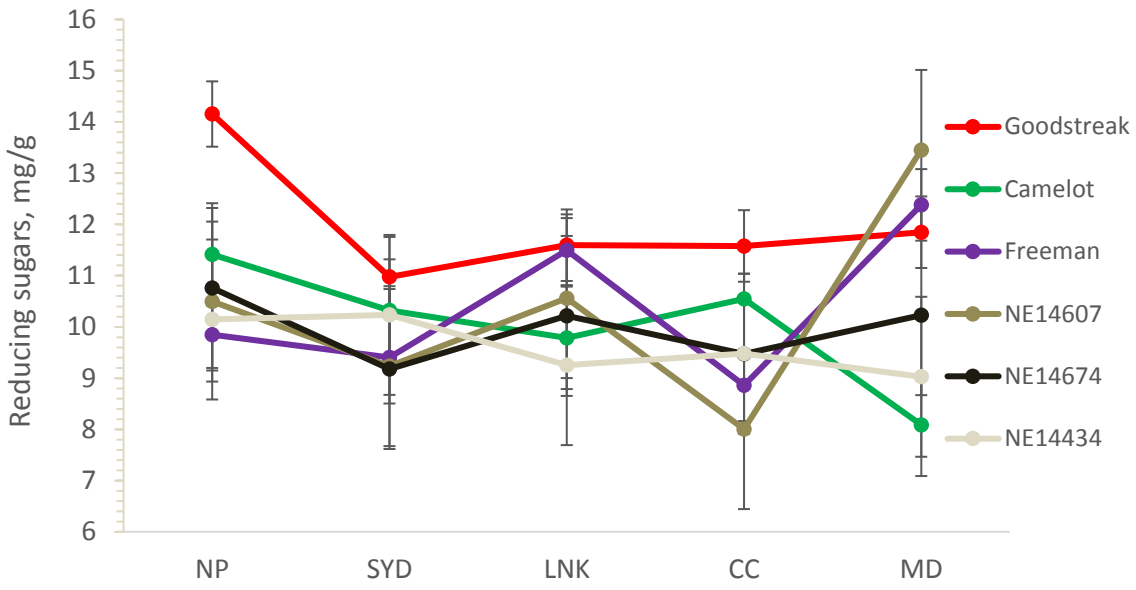

Figure 11 - Variation of reducing sugars concentration among locations and cultivars

3.5 SKCS data analysis

Hardness, size and weight of kernels were evaluated among cultivars and locations. Hardness had a significant G x E interaction (p=0.0104); however, it was clear that the interaction was significant due to change in magnitude and not crossover effects (Fig. 12). These results indicate that hardness is mostly driven by genetics as was previously reported [16]. NE 14607 had the highest hardness index and Freeman along with NE 14434 had the lowest.

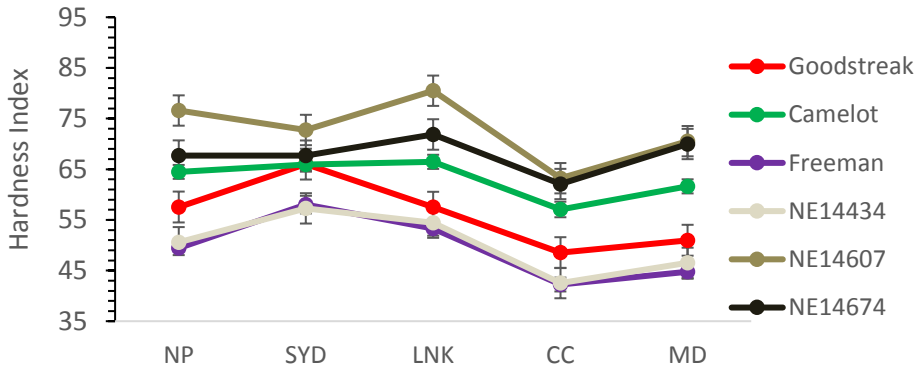

Figure 12 - Variation of kernel hardness among cultivars and locations

A significant G x E interaction was also recorded for kernel size (Table 3). The diameter of measured kernels varied from 2.5 to 2.9 mm, and Camelot consistently had the highest values of kernel diameter except in North Platte (Fig. 13).

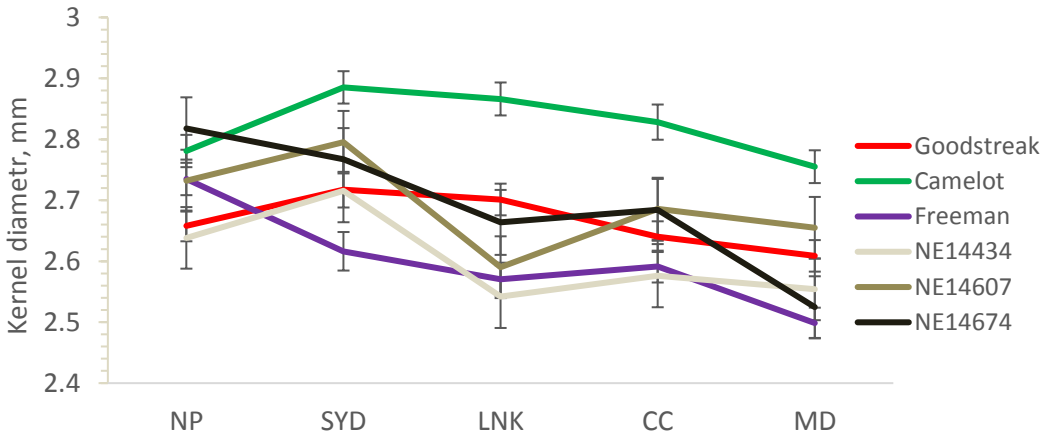

Figure 13 - Variation in kernel weight among the locations and cultivars

Kernel weight showed significant a G x E interaction (Table 3). Kernel weight varied among cultivars and locations within the range of 27-40 mg. Cultivar Camelot tended to have the highest kernel weight. This aligns with our findings on kernel diameter which also showed that Camelot was superior over

the other cultivars among all of the locations. Most kernels had higher weight in Sydney; only Freeman and NE14674 were not highest in this location (Fig. 14).

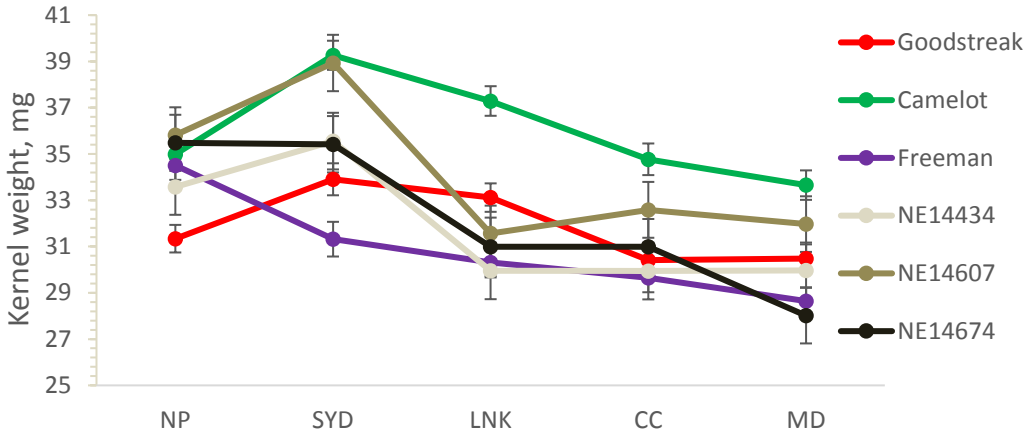

Figure 14- Variation in kernel weight among the locations and cultivars

3.5.1 Yield data

Only the location effect was significant for yield (Table 3). Lower yields were recorded in North Platte and Mead, which had the longest period from flowering till harvest: 45 days (see data in appendix). In contrast, Lincoln, which had the shortest period between flowering and harvest: 26 days, had the highest yield. This can be explained by the higher rate of kernel abscission in North Platte due to the expanded harvest period (Fig. 15).

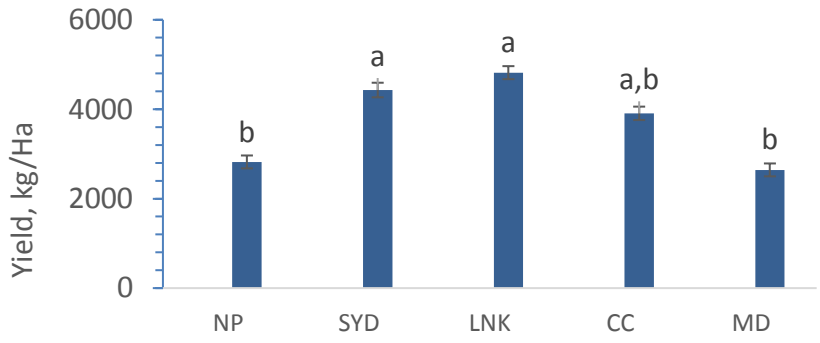

Figure 15- Variation in yields among the growing locations

3.6 Correlations between the measured variables based on data from 2014

Correlations were calculated among the response variables (Table 5). Asparagine had a significant positively correlated with alanine and total protein. This correlation is easy to explain as far as all these parameters describe protein content of the grain and increase of one variable associated with increase of another.

Table 5. Correlation between measured variables; the right part of the table shows correlation coefficients (red=positive; blue=negative); the left part of the table shows p-values (yellow=p<0.01; green=p<0.05)[A].

r/p	Ala	Asn	FAA	HMW	Hardness	LMW	PR	Protein	RedSug	Size	Weight	Yield
Ala		0.662	0.170	-0.143	0.276	0.143	-0.172	0.314	0.121	0.115	0.182	-0.465
Asn	<.0001		0.337	-0.248	0.153	0.248	-0.256	0.517	0.281	0.367	0.374	-0.321
FAA	0.3362	0.051		0.291	-0.036	-0.291	0.292	-0.181	0.085	0.087	0.201	0.133
HMW	0.4209	0.1573	0.0945		-0.490	-1.000	0.977	-0.558	-0.250	-0.309	-0.152	0.159
Hardness	0.1197	0.3954	0.8419	0.0038		0.490	-0.562	0.087	0.109	0.354	0.411	0.194
LMW	0.4209	0.1573	0.0945	<.0001	0.0038		-0.977	0.558	0.250	0.309	0.152	-0.159
PR	0.3304	0.1446	0.0937	<.0001	0.0007	<.0001		-0.545	-0.283	-0.327	-0.190	0.146
Protein	0.0709	0.0017	0.3046	0.0006	0.6289	0.0006	0.0009		0.368	0.179	-0.020	-0.554
RedSug	0.4959	0.1075	0.6332	0.1533	0.5447	0.1533	0.1051	0.0321		-0.186	-0.202	-0.368
Size	0.5233	0.0357	0.6286	0.08	0.0435	0.08	0.0629	0.3184	0.301		0.916	0.246
Weight	0.3121	0.0318	0.2611	0.3988	0.0176	0.3988	0.2895	0.9135	0.2603	<.0001		0.353
Yield	0.0056	0.0642	0.4538	0.3684	0.28	0.3684	0.4105	0.0007	0.0325	0.1681	0.0438	

[A]Ala – alanine, Asn – asparagine, FAA – total free amino acids, HMW – high molecular weight proteins, LMW – low molecular weight proteins, PR – protein ratio (HMW/LMW), RedSug – reducing sugars, Size – kernel size, Weight – kernel weight.

There was a negative correlation between asparagine and yield. As mentioned, lower yields in North Platte can be explained by the longer period from flowering to harvest which led to abscission of kernels. On the other hand, Lincoln had the shortest period between flowering and harvest and the highest yields. Therefore, longer periods between flowering and harvest can contribute to drought stress which leads to increase in asparagine concentration.

Our study also showed significant positive correlations between the level of asparagine and seed size and weight. Notably, the other measured variables such as alanine or reducing sugars do not show any significant correlation with kernel size or weight. Moreover, even after adjustment on protein level (Table 6) these correlations still appear to be close to being significant (p=0.0692 and 0.011, respectively). So we can report that higher level of asparagine associated with increase of kernel size but more biological evidence and larger sample set is needed to support this hypothesize.

Table 6. Correlations between measured parameters with protein adjustment; the right part of the table shows correlation coefficients (red=positive; blue=negative); the left part of the table shows p-values (yellow=p<0.01; green=p<0.05)[A]

r/p	Ala	Asn	FAA	HMW	Hardness	LMW	PR	RedSug	Size	Weight	Yield1
Ala		0.6153	0.26967	0.04408	0.26308	-0.04408	0.0005	0.0005	0.06513	0.19665	-0.36819
Asn	0.0002		0.57453	0.06478	0.12672	-0.06478	0.04172	0.10152	0.32539	0.44357	-0.04652
FAA	0.1355	0.0006		0.21658	-0.02619	-0.21658	0.2287	0.24353	0.11085	0.2005	0.03458
HMW	0.8107	0.7247	0.2338		-0.52766	-1	0.96773	-0.04318	-0.25645	-0.19317	-0.22018
Hardness	0.1457	0.4895	0.8868	0.0019		0.52766	-0.61054	0.08527	0.34485	0.41415	0.28843
LMW	0.8107	0.7247	0.2338	<.0001	0.0019		-0.96773	0.04318	0.25645	0.19317	0.22018
PR	0.9978	0.8207	0.208	<.0001	0.0002	<.0001		-0.09669	-0.27865	-0.23648	-0.22588
RedSug	0.9978	0.5803	0.1792	0.8145	0.6427	0.8145	0.5986		-0.26521	-0.20718	-0.21087
Size	0.7232	0.0692	0.5458	0.1565	0.0532	0.1565	0.1225	0.1424		0.93508	0.41547
Weight	0.2807	0.011	0.2712	0.2895	0.0184	0.2895	0.1925	0.2552	<.0001		0.40813
Yield1	0.0381	0.8004	0.851	0.2259	0.1094	0.2259	0.2138	0.2467	0.018	0.0204	

[A]Ala – alanine, Asn – asparagine, FAA – total free amino acids, HMW – high molecular weight proteins, LMW – low molecular weight proteins, PR – protein ratio (HMW/LMW), RedSug – reducing sugars, Size – kernel size, Weight – kernel weight.

HMW and LMW protein as well as PR showed significant correlations with hardness and total protein. Hardness positively correlated with seed size on a level which approached significance (p=0.0532). This result indicated that protein level correlates positively with LMW proteins, which suggests that under conditions of protein excess, grains tend to accumulate LMW proteins instead of HMW proteins. Considering the relationship between friabilin protein

(MW=15kDa) [17], and kernel hardness, we can conclude that our study supports the idea that kernel hardness is driven by LMW proteins based on the significant positive correlation between those two parameters.

Our study showed a negative correlation between yield and asparagine. Moreover, we suggest that lower yields (2822.01 kg/Ha) can be explained in North Platte by longer period from flowering to harvest (45 days) which leads to abscission of kernels, on the other hand Lincoln has the shortest period between flowering and harvest (26 days) and the highest yields – 4818.3 kg/Hg. Moreover, longer period between flowering and harvest can contribute to drought stress which leads to increase in asparagine concentration. Based on that, correlation between asparagine level and yields can be drawn.

3.7 Asparagine concentration in samples from 2016.

Results from 2016 showed significant G x E interaction ($p=0.0076$) on asparagine concentration, location ($p<0.0001$) and cultivar ($p<0.0001$) effects were also significant. Overall samples from 2016 had higher level of asparagine compared to samples from 2014. Disease outbreak can explain the higher level of asparagine in Mead in 2016 (Fig. 16) [18]. We assume that the level of asparagine can be triggered by changes in management practices or environment conditions. Cultivar Goodstreak showed high values of asparagine among cultivars (Fig. 16). This cultivar had a high level of asparagine compared to other lines in 2014 as well (Fig. 9).

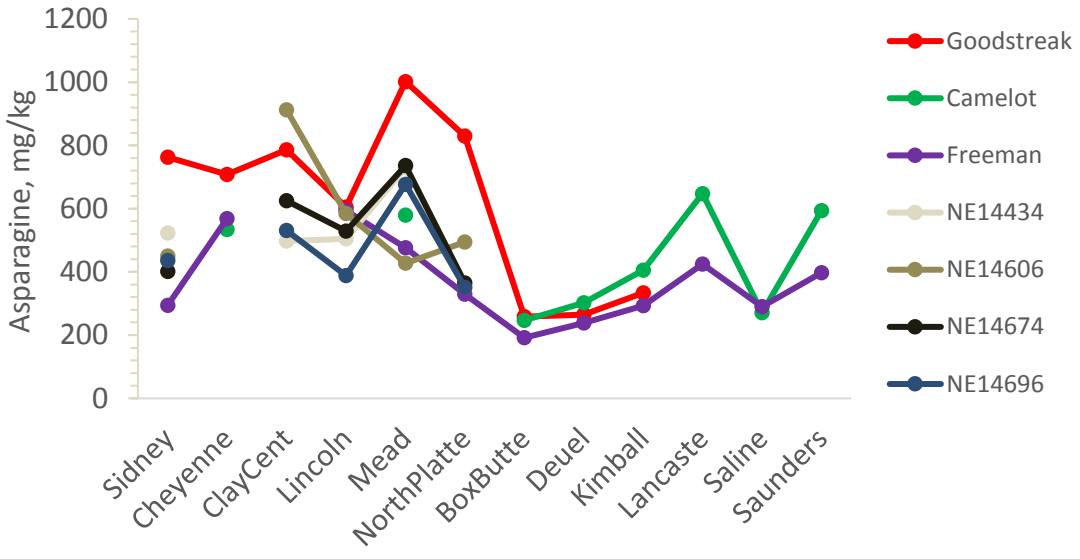

Figure 16 - Variation in asparagine concentration among the locations and cultivars in 2016

3.8 Conclusions

In 2014, North Platte had a higher level of asparagine compared to other growing locations in Nebraska. We suggest that higher level of asparagine can be caused by sulfur deficient soil or longer period between flowering and harvest. We also found correlation between kernel size, weight and asparagine level which was not previously reported in the literature. Finally, the correlation between lower yields in North Platte and higher asparagine level at this location was potentially explained by longer grain-filling period.

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4 Chapter 4. Variation in free asparagine in proso millet

4.1 Abstract

The rise in popularity of gluten-free foods has opened up opportunities for underutilized grains. Proso millet, an important grain produced in western Nebraska, is one such grain that is becoming more popular for human food. The objective of this study was to determine the concentration of asparagine in proso millet compared to wheat and to determine the effect of artificial irrigation on asparagine concentration in proso. The concentration of asparagine varied from 60-150 mg/kg and, on average, was almost three times lower compared to wheat. In addition, artificial irrigation did not significantly change asparagine concentration; however, a longer period between flowering and harvest led to an increase in asparagine concentration (as was also the case for wheat).

4.2 Introduction

Due to the high public attention to Celiac disease and the growing interest in gluten products, production of gluten-free grains is growing by 20% annually [1]. This has led the food industry to increase the use of gluten-free flours to produce baked products, which may have increased (or decreased) potential for acrylamide formation compared to wheat-based products. There are very few studies on the genetic and environmental variation in asparagine concentration in gluten-free grains. Curtis et al. [2] reported a variation in free asparagine of 450-1100 mg/kg in rye, which suggests similar results compared to our study on wheat (200-1100 mg/kg) but slightly lower compare to result reported by Corol et

al. [3] (320-1500 mg/kg), which utilized broader scope of wheat varieties. Therefore, this area of research needs further development.

Preliminary data on asparagine, as a marker for acrylamide-forming potential, was collected on a convenience sample set of different grains that may be used in gluten-free baking (Table 7). Oats and pearl millet had higher asparagine compared to the average for wheat obtained in our previous study (see Chapter 3), however, red and white sorghum and especially proso millet showed lower asparagine. Based on these data we hypothesized that proso millet has significantly lower asparagine compared to wheat.

Table 7. Asparagine and alanine concentrations (mg/kg) in a convenience sample of different grains.

	Wheat		Sorghum		Millet	
Amino acid	(average)	Oats	Red	White	Proso	Pearl
Asparagine	247	296	112	152	83	779
Alanine	135	197	108	52	63	357

Proso millet is a major food source in many countries, including northern China, India, southern African countries, and Russia [4, 5]. In the United States, however, it is used primarily to replant fields of winter wheat or winter barley lost to winterkill, soil erosion, drought and other agronomic practices [6, 7]. The grain is used as livestock feed and in birdseed mixtures [8,9].

It is well known that proso is a drought resistant crop [10]. However, no data exists on the effect of artificial irrigation on asparagine accumulation in proso millet. It was previously reported in literature that proline and asparagine concentration increase in plats, which have been subject to drought stress: soybean [11], pearl millet [12], wheat [13] Because of its drought tolerance, we

hypothesize that additional artificial irrigation will not affect the level of asparagine in proso; rather, asparagine concentration will vary based on genetic or environment effects and their interaction. Lower asparagine would be important to Nebraska, since proso millet is an emerging crop in this state [14].

4.3 Materials and methods

4.3.1 Experimental design

To test our hypotheses, we performed a study on 13 proso millet genotypes: Cope, Dawn, Earlybird, Horizon, Huntsman, Minco, Minsum, Panhande, Plateau, Rise, Snobird, Sunrise, Sunup. These cultivars were planted in irrigated and dryland trials at Sidney, Nebraska. The sample set did not include all of the varieties and replicates in both trials. In 2013, Dawn, Horizon, Huntsman and Sunrise were replicated in both trials. In 2014, Earlybird, Horizon, Huntsman, Minco, Sunrise and Plateau were included in both trials. In 2015, Sunup, Sunrise, Snobird, Earlybird and Horizon were replicated in both trials. Other varieties were included either irrigated or dryland trials. Samples from 2013 had 1-2 replicates; 2014 had 2-4 replicates; 2015 had 1-2 replicates. Detailed information regarding data set introduced in table 8.

Table 8. Number of replicates for each cultivar of proso millet across growing years and locations

	Irrigated trial			Dry trial		
Cultivar	2013	2014	2015	2013	2014	2015
Sunrise	1	4	2	1	2	1
Horizon	1	2	2	1	2	1
Dawn	1	4	0	2	0	1
Plateau	1	4	2	0	2	1
Huntsman	1	4	2	2	2	1
Sunup	0	0	2	2	2	1

Earlybird	0	4	2	2	2	1
Minco	0	3	2	0	2	1
Minsum	0	2	0	0	0	0
Panhandle	0	0	0	0	2	0
Cope	0	0	0	0	2	0
Rise	0	0	0	0	2	0
Snobird	0	0	2	0	0	1

4.3.2 Asparagine detection

Asparagine level was determined using NMR spectroscopy as described previously (see Chapter 3).

4.3.3 Statistical analysis

Data was analyzed using GLMMIX for all three variables year, irrigation, and cultivar. After that data was analyzed on each year separately based on two factors: cultivar and irrigation. Significant differences among least squares means were calculated based on Fisher's least significant difference ($\alpha < 0.05$).

4.4 Results and discussions

When statistical analysis was performed based on 3 factors: year, cultivar and irrigation type all factors and interactions were significant (table 9) except irrigation effect. The highest asparagine level was recorded in 2014 (Fig. 17). Due to the significant 3-way interaction, I performed ANOVA by year to determine asparagine level among the cultivars in each separate year.

Table 9. ANOVA for asparagine concentration in proso samples

Analysis	Source of variance	Df	Asparagine
Full	Year	2	5870.5*
	Cultivar	12	1421.0*
	Year*Cultivar	13	350.0*
	Irrigation	1	93.0
	Year*Irrigation	2	381.8*
	Ciltivar*Irrigation	8	230.0*

	Year*Cultivar*Irrigation	7	285.9*
	Error df	33	
	Mean Square Error		36
Year 2013	Cultivar	7	149.7*
	Irrigation	1	327.3*
	Cultivar x Irrigation	3	24.3
	Error df	5	
	Mean Square Error		5
Year 2014	Cultivar	11	808.8*
	Irrigation	1	1.50
	Cultivar x Irrigation	5	100.2*
	Error df	7	
	Mean Square Error		25
Year 2015	Cultivar	8	454*
	Irrigation	1	590*
	Cultivar x Irrigation	7	224*
	Error df	8	
	Mean Square Error		6

* Numbers showed in asparagine column represent Mean Squares and those marked with asterisks "*" significant at $p \leq 0.05$.

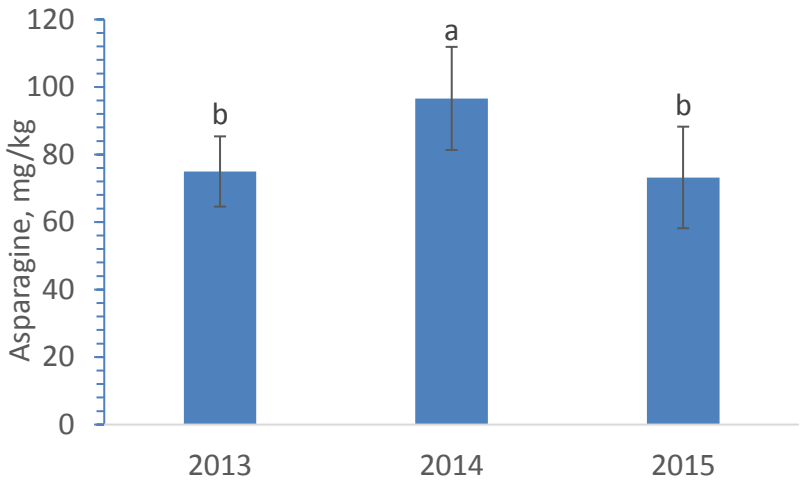

Figure 17 - Variation in asparagine concentration among the years

Finally, collected data on proso millet samples support our hypothesis that proso millet flour contains less asparagine compare to wheat flour. Average proso sample which was analyzed in our study has the concentration of asparagine 85.5 mg/kg when the average amount of asparagine in wheat flour from our study is 271 mg/kg (Fig. 18).

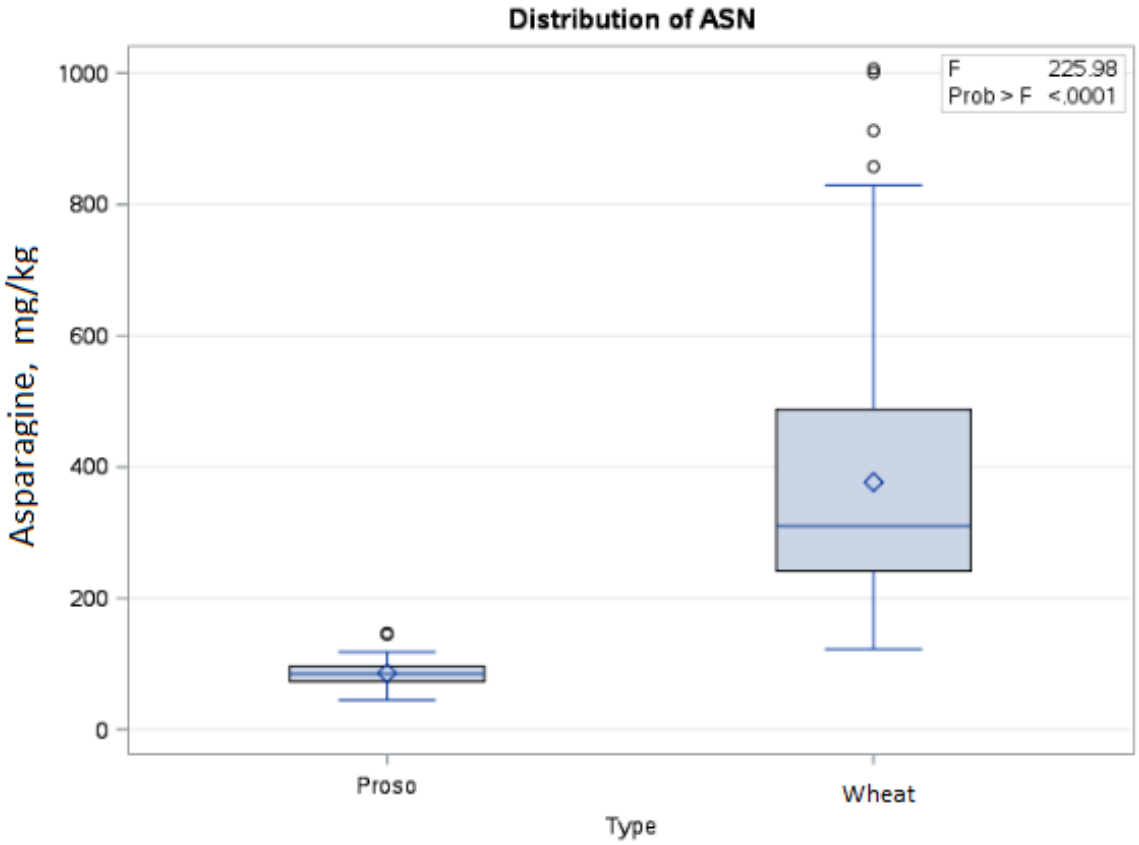

Figure 18 - Asparagine concentration in wheat and proso millet (error bar represent the highest values)

4.5 Analysis of asparagine level in proso samples from 2013.

There was not a significant interaction between irrigation type and cultivar in 2013 (Table 9). Therefore, we can interpret main effects. Fig. 19 represents main effect of irrigation type. The irrigated trial had higher concentration of asparagine. Among the cultivars, Dawn, Plateau and Hantsman showed the highest asparagine concentration and Earlybird in dryland conditions had the lowest (Fig. 20).

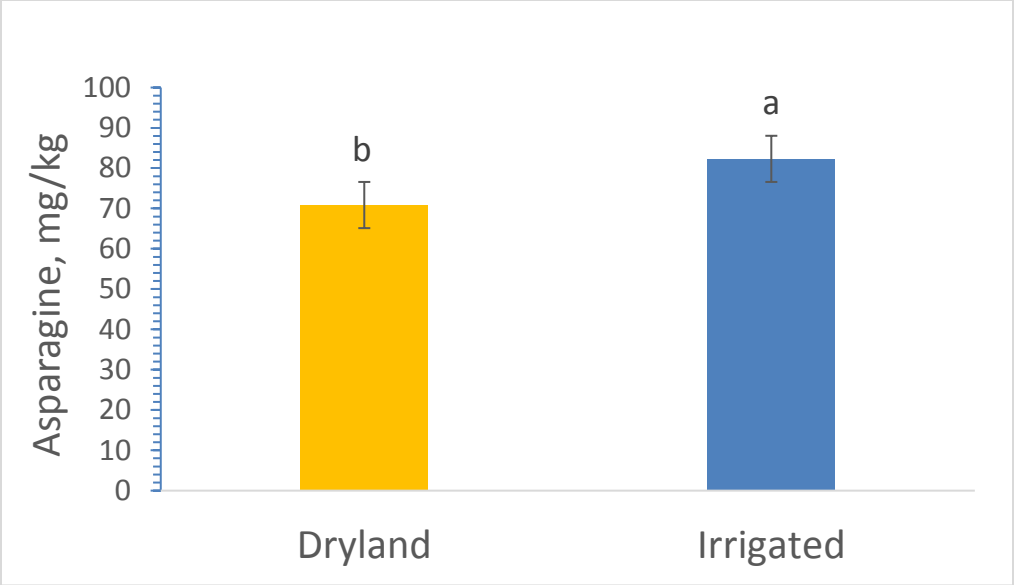

Figure 19 - Effect of irrigation on asparagine concentration in proso samples from 2013

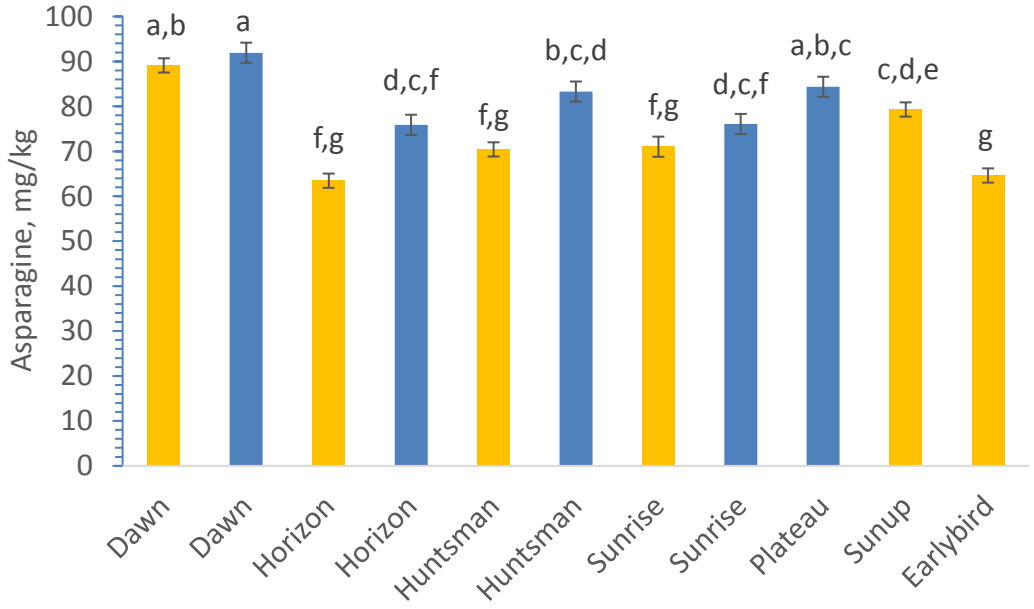

Figure 20 - Variation in asparagine among proso cultivars in 2013
Cultivars that were grown with artificial irrigation are colored blue; yellow represents the dryland trial.

Collected meteorology data (Table 10) indicated that cultivars of proso from irrigated trial have longer period from flowering to harvest, which led to higher total radiation and evaporation. This is in accordance with our conclusions

on wheat samples from 2014 which indicated higher asparagine in locations that had longer period between flowering and harvest.

Table 10. Meteorology data from 2013.[A]

Cultivar/trial	Flowering date	Days to harvest	Temp (°C, avg)	Rh (%, avg)	Soil Temp (°C, avg)	Precipitation* (mm/d, avg)	Precipitation* (mm, total)	Solar Rad (MJ/m^2, avg)	Solar Rad (MJ/m^2, total)	ET (mm/d, avg)	ET (mm, total)
Sunrise											
Dryland	4-Aug	79	17.0	61.6	18.7	3.16	250	16.3	1288	5.54	438
Irrigated	31-Jul	95	16.1	61.8	17.6	2.65	252	16.0	1519	5.36	509
Dawn											
Dryland	28-Jul	86	17.2	62.6	19.0	2.96	255	16.4	1413	5.52	475
Irrigated	30-Jul	96	16.1	61.8	17.7	2.63	252	16.1	1544	5.38	516
Horizon											
Dryland	1-Aug	82	17.1	62.1	18.9	3.08	252	16.3	1339	5.55	455
Irrigated	2-Aug	93	15.9	61.8	17.5	2.71	252	15.9	1477	5.31	494

[A]Temp, temperature; avg, average; Rh, relative humidity; Rad, radiation; ET, evapotranspiration.
*Data on precipitation relates only to natural precipitation and do not include artificial irrigation.

Therefore, data from 2013 showed that samples which were grown with additional artificial irrigation have a higher level of asparagine compare to the naturally irrigated trial. In 2013 all four proso cultivars (Dawn, Sunrise, Horizon, Huntsman), which were replicated in both trials, had higher level of asparagine in irrigated trail (Fig. 20).

4.6 Analysis of asparagine in proso millet from 2014

There was a significant interaction between irrigation type and cultivar in 2014. Irrigation by itself was not significant, but cultivar was significant (Table 8). Cultivar Cope from the dryland trial had the highest asparagine concentration

and Earlybird had the lowest asparagine in both dryland and irrigated trials (Fig. 21).

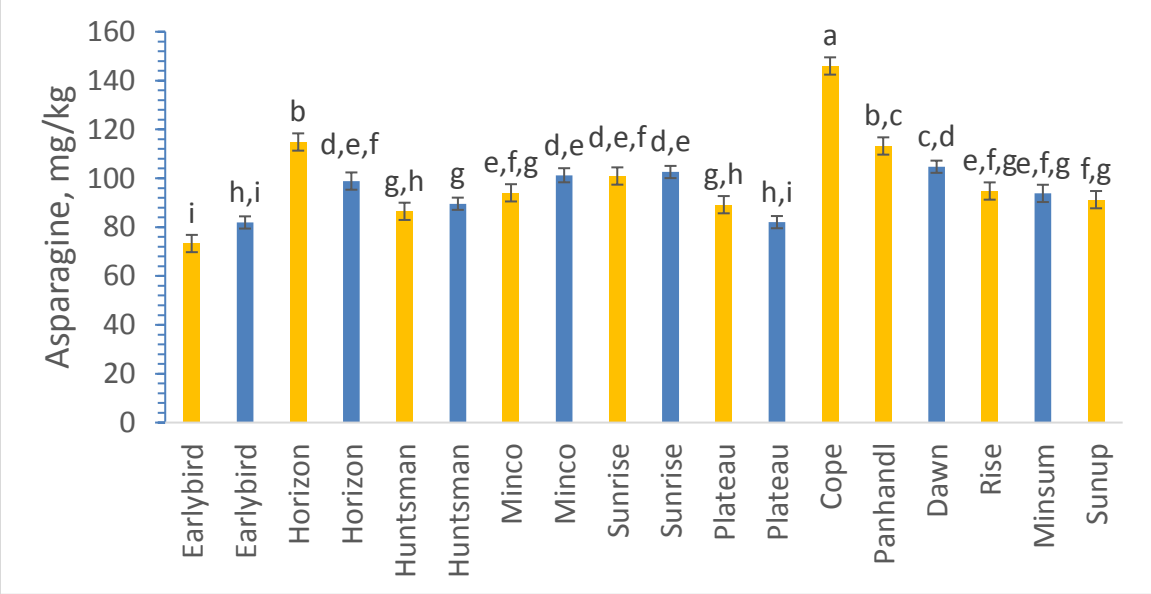

Figure 21 - Variation among cultivars with and without artificial irrigation in 2014

Data from 2014 showed that only Horizon and Plateau had higher level of asparagine in dryland trial, while Minco, Huntsman and Earlybird showed opposite results (Fig. 21).

4.7 Analysis of asparagine level in proso samples from 2015

Data from 2015 showed similar results to those that were obtained in 2014. All of the interactions and simple effects were significant (Table 8). Snobird showed the highest asparagine concentration in the dryland trial, meanwhile Huntsman had the lowest asparagine concentration under dryland conditions (Fig. 22). Some of the cultivars changed ranking on asparagine level among the years, for example Horizon was among the lowest cultivars in 2013 but in 2014 it

was one of the highest. Meanwhile Huntsman had the lowest level of asparagine in 2015 as well as one of the lowest in 2013. Finally, Earlybird variety consistently had the lowest concentration of asparagine across all three years.

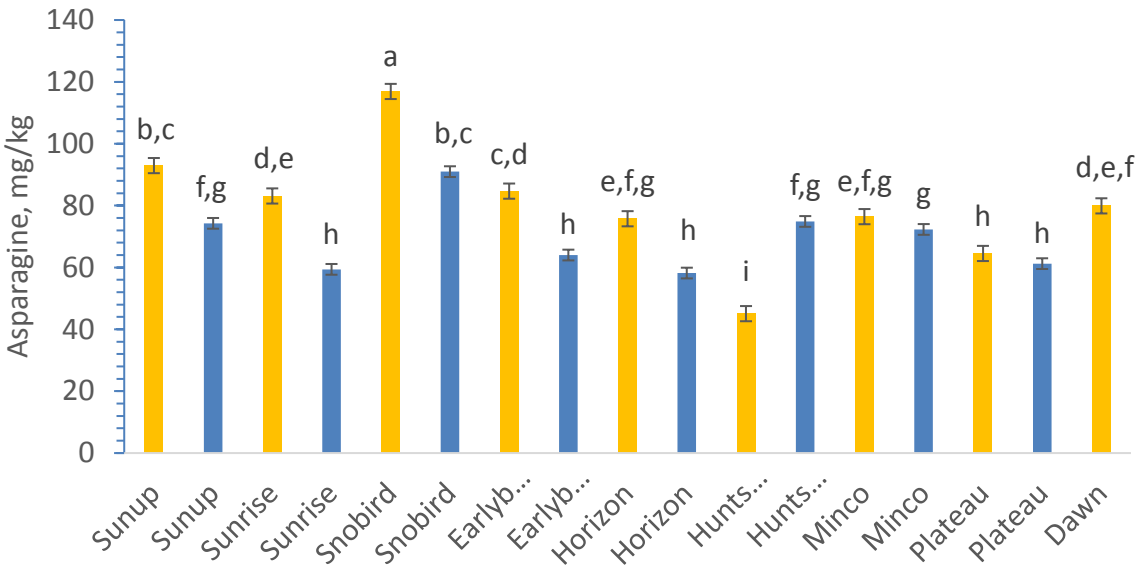

Figure 22 - Variation among cultivar lines with and without artificial irrigation in 2015

2015 Snobird from dry trail had the highest level of asparagine among other studied cultivars. In addition, all cultivars except Huntsman had higher level of asparagine in dry trail compare to irrigated trial.

4.8 Conclusions

Results from our study showed that proso millet has almost three times lower concentration of asparagine compare to wheaton average, which supports out hypothesize from the preliminary screening of asparagine level in different grains. We also can confirm our hypotheses that lack of artificial irrigation does not tend to increase the concentration of asparagine. Meanwhile, studied

varieties changing the ranking based in asparagine concentration, but Earlybird tends to constantly have the lowest concentration of asparagine in all years.

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5 Chapter 5. Introduction of asparaginase activity in wheat

5.1 Abstract

The first report of the potential presence of asparaginase in wheat will be discussed in this chapter. This assumption was made based on the decrease of asparagine concentration measured by NMR spectroscopy during an extraction and simultaneous increase of ammonia observed by UV spectroscopy. These data suggest that asparaginase is naturally present in the wheat kernel. In addition preliminary data was collected on optimal conditions of activity for this enzyme.

5.2 Introduction

Asparaginase has been used to reduce asparagine and ultimately acrylamide formation in baked products [1]. The studies [2,3] show that level of asparagine can be decreased in dough by adding yeast. Collar et al. [3] reported decrease in asparagine concentration during the fermentation with yeast by 92%, meanwhile fermentation with lactic acid showed a decrease of 60%. This enzyme catalyzes the hydrolytic cleavage of the amino acid L-asparagine to L-aspartic acid and ammonia (Fig. 23).

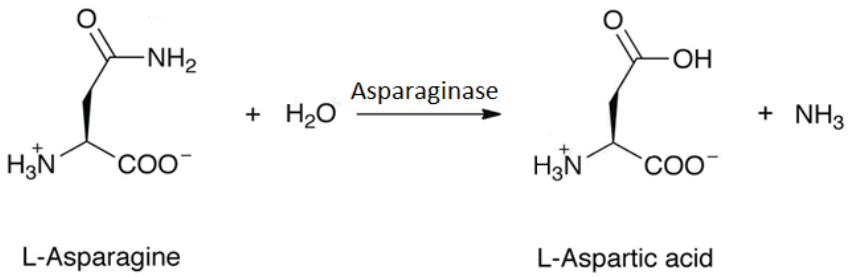

Figure 23 – Scheme of asparaginase activity [2]

Therefore, one of the most efficient methods of reducing acrylamide formation is the addition of L-asparaginase to dough during the mixing stage. Previous research [4] has shown that L-asparaginase has the ability to reduce the formation of acrylamide in the range of 23-75 % depending on the pH of dough and time of enzyme incubation.

L-Asparaginase is found in numerous microorganisms with different activities: *Aeromonas hydrophila* (40-150 units/gram of cells), *Erwinia aroideae* (55-770), *Escherichia coli* (0-225), *Proteus americanus* (160), *Pseudomanas acidovorans* (175-210), *Bacillus subtilis* (0-55) [5]. Some of the microorganism which have asparaginase activity might be present in wheat flour. For example Berghofer et al. [6] reported presents of *Escherichia coli* and *Bacillus ssp*.in wheat flour which have asparaginase activity according to Pettersen et al.[5].

Asparagine in plants can be used as a nitrogen storing amino acid, due to its high N:C ratio (2:4) [7]. Four days of maintaining the plants in nitrogen-free conditions caused a reduction in the proportion of asparagine and an increase in aspartate in all the plants tested [8]. This data pointing out that plants have an efficient mechanism of converting asparagine into aspartate, and the most effective way of doing that is by means of *L*-asparaginase.

A host of enzymes have been reported in wheat flour: amylases, xylanase, protease, lipase, etc.; however, the presence of asparaginase has not previously been reported [9].

We hypothesize that in whole grain products acrylamide formation is a function of both asparagine concentration and asparaginase activity. While others have reported that asparagine concentration alone is the key determinant of acrylamide generation in finished products [10], these have been in refined flour products. Furthermore, if asparagine were the only determinant of acrylamide concentration in finished products we would expect far higher acrylamide concentrations in whole wheat breads compared to white breads, since asparagine is concentrated in the bran fraction of the kernel [11]. However, this does not seem to be the case (Table 11). Furthermore, because most enzymatic activity is present in the outer layers of the wheat kernel (germ, aleurone, pericarp), we anticipate that asparaginase activity is also located in the bran fraction.

Table 11. Acrylamide content of white and whole grain breads [11]

Sample	Acrylamide (ppb)
White breads	
Pepperidge Farm	36
Sunbeam	18
Enriched	
Freihofer's	30
Kroger	35
Whole wheat breads	
Arnold Bakery	102
Freihofer's	25
Natural Ovens	44
Valley Grains	45

Therefore, more research is needed to evaluate potential presence of an *L*-asparaginase in wheat and other grains. Precise evaluation of activities of this

enzyme can help to modify baking conditions to eliminate risk of acrylamide formation.

5.3 Materials methods

5.3.1 Asparagine quantification

Determination of asparagine was performed according to Baker et al. [12] with same modifications which were discussed in Chapter 2. However, extraction temperature in experiments which will be introduced in this chapter was 50 °C.

5.3.2 Asparaginase activity

Asparaginase activity was measured by the generation of ammonia. For the measuring amount of released ammonia, 200 mg of whole grain flour were weighed into a 2 mL microfuge tube. Another tube was prepared for stopping the reaction by adding 0.1 mL of 1 M HCl to a clean 2 mL microfuge tube. In some experiments, additional amount of asparagine (50 µmol) was added for testing asparaginase activity in the presence of substrate abundance.

Dry sample was vortexed to aerate it and 1 mL of water or buffer was immediately added, capped, and vortex mixed. Buffers used included: sodium acetate (pH 5, 50 mM) and tris-HCl (pH 8, 50 mM).

Then the tube was placed in a water bath at 37 °C. Tubes were secured horizontally in the water bath at 150 rpm. After specified time periods, 0.1 mL of slurry was withdrawn with a pipette tip with the end cut off and immediately mixed with the prepared tubes containing 0.1 mL of 1 M HCl.

When all tubes were collected, they were centrifuged at max speed (15000 rpm) for 10 min. 0.1 mL of the supernatant was removed to a fresh tube and diluted to 1 mL (by adding 0.9 mL water).

After that, samples were derivatized for ammonia quantification. 40 µl of phenol-alcohol solution (prepared by mixing 1 mL of liquefied phenol and 9 mL of absolute ethanol) was added to the diluted sample. The test tube was closed and vortexed for 6 s. 40 µl of 0.5% sodium nitroprusside in water was then placed into the same tube and vortexed for 6 s.

0.1 mL of freshly prepared oxidizing solution (prepared by mixing 4 parts alkaline solution with 1 part of commercially available liquid bleach) was then added. The alkaline solution was prepared by dissolving 10 g of trisodium citrate and 0.5 g of sodium hydroxide in 50 ml of water. After addition of oxidizing solution, sample tube was vortexed for 6 s.

The color was developed for 1 h at room temperature and then absorbance readings were recorded at 640 nm. Ammonia was quantified by means of external calibration with ammonium chloride (1-10 µM ammonia, which is ~0.05-0.5 mg ammonium chloride/L).

5.3.3 Evaluation of asparagine level in dough during fermentation

135 ml of water was added to 180 g of whole wheat flour; 2.2 g of yeast was added to yeast containing trial [13]. The dough was then kneaded by hand for 5 min. Samples of dough were pinched off every 10 min and placed into the freezer (-18 °C). After that, all samples were freeze-dried. 30 mg of freeze-dried

solids were then placed into a 2 ml tube. After that, 800 µl of deuterated water and 200 µl of deuterated methanol were added. Samples were vortexed and placed at a room temperature for 1 hour.

Samples were then centrifuged and 400 µl of supernatant was placed into 5 mm NMR tube which contained 60 µl of 0.293 mg/ml TSP solution in 80 D_2O: 20 d-Methanol.

5.4 Results and discussion

The first clue to asparaginase activity in wheat samples was collected during the extraction of asparagine for the study described in Chapter 2, which was carried out according to Baker et al. [12]. To ensure complete extraction of the asparagine, extraction time was studied. A longer extraction time (50 °C, 150 rpm) led to a decrease in measured asparagine concentration (Fig. 24). Data available in the literature suggested that asparaginase can retain significant activity even at 50 °C (Fig. 25) [14]. Therefore for further analysis of asparagine, extraction conditions were changed to 10 min at 90 °C, which eliminated the decline in asparagine. We obtained the result of 189 mg/kg during the 10 min extraction at 90 °C. This result was comparable to data presented on Fig. 24, which, by extrapolation to time zero would be a concentration of 182 mg asparagine/kg. These data suggested significant asparaginase activity, which would be able decrease the amount of asparagine during processing.

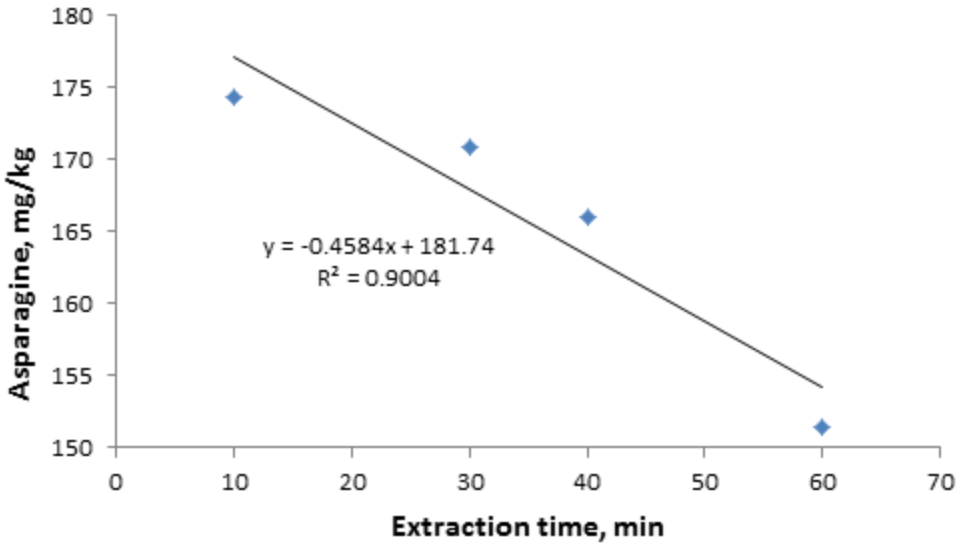

Figure 24 - Decline of asparagine concentration during the extraction

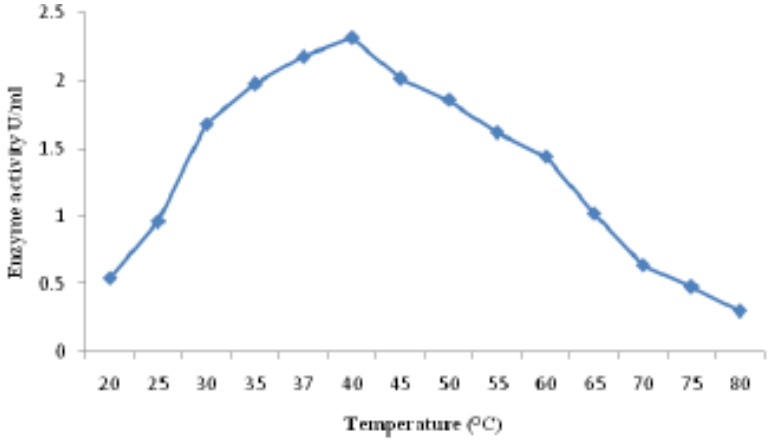

Figure 25 - Effect of temperature on the activity of purified L-asparaginase from *F. Culmorum* [14]

Further experiments were carried out on two different cultivars of hard red winter wheat: Freeman and Anton, to confirm the presence of asparaginase. This time, the approach was to measure ammonia evolution, a product of asparaginase activity. Ammonia increased during the reaction in a non-linear fashion as would be expected for an enzymatic reaction (Fig. 26). The two cultivars analyzed had different rates of ammonia accumulation during

incubation, with Freeman showing substantially higher ammonia production. This provided further evidence that asparaginase is naturally present in the wheat kernel.

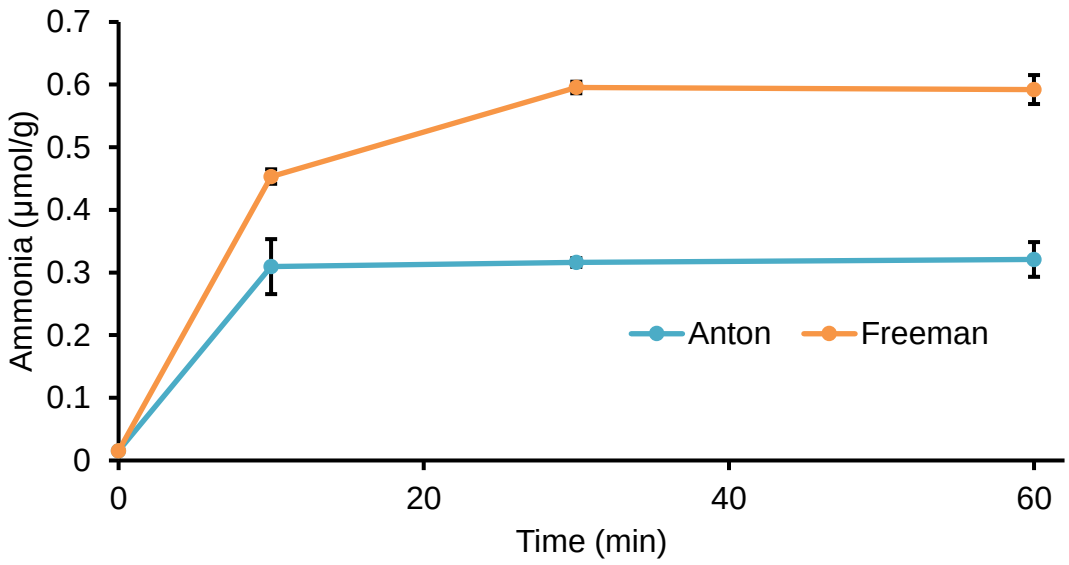

Figure 26 - Ammonia accumulation during extraction of asparagine

In a follow-up experiment, asparaginase activity was tested at pH 5 and pH 8 using Freeman wheat as the source of asparaginase. pH 8 was selected because this is the optimum pH for many microbial asparaginases [14]. pH 5 was selected because this is an optimum pH for many enzymes in wheat [15]. Performing the reaction at pH 5 resulted in higher evolution of ammonia compared to pH 8 (Fig. 27). These data suggested 1) that the asparaginase activity may be of endogenous origin, rather than exogenous (microbial) and 2) that endogenous asparaginase activity could play a role in decreasing asparagine during food processing since pH 5 is close to the pH experienced in many wheat-based foods.

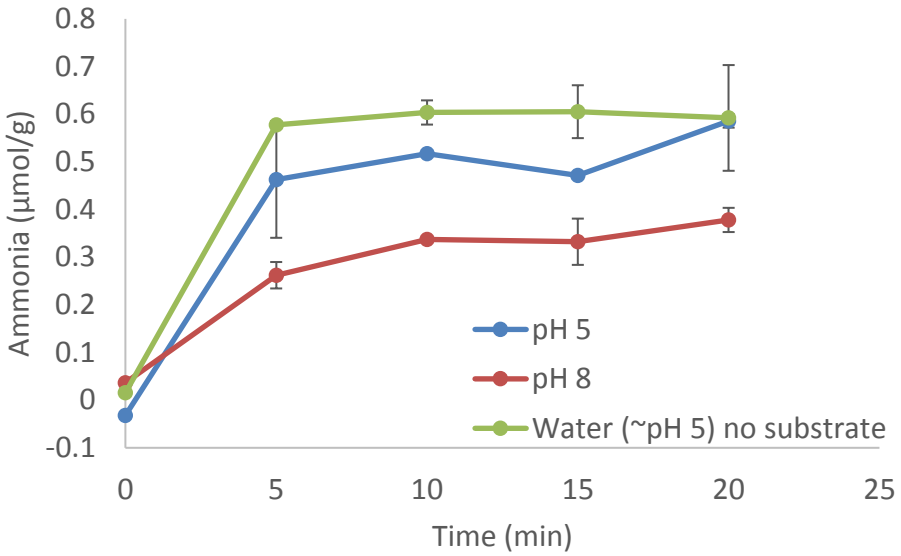

Figure 27 - Testing of enzyme activity in different pH, with and without additional substrate addition

Asparaginase activity was also tested with and without an addition of additional amount of substrate (asparagine). An additional amount of substrate did not increase the amount of ammonia released, even slight inhibition was observed. After converting the amount of released ammonia to asparagine we can conclude that almost 20% of asparagine was transformed by asparaginase during incubation.

5.5 Reduction of asparagine concentration in dough during fermentation

Fredriksson et al. [2] reported a significant decrease (up to 87%) during 6 h of wheat dough fermentation. Such significant decrease of asparagine was explained by metabolism by yeast [16]. However the same author reported only 30% decrease of asparagine during 72 h of sourdough fermentation, which can be explained by low enzyme activity at pH=3.7 [2].

However, our data suggested that the decrease in asparagine may be caused by the activity of endogeneous asparaginase. Therefore we prepared wheat dough with and without yeast. During fermentation, both doughs showed decreases in asparagine (Fig. 28). In the dough without yeast, the concentration of asparagine decreased from 248 mg/kg to 158 mg/kg, which was more dramatic than with yeast, which decreased to 204 mg/kg. These data support the idea that decrease in asparagine concentration in dough is due to asparaginase contained in flour and not due to yeast metabolism.

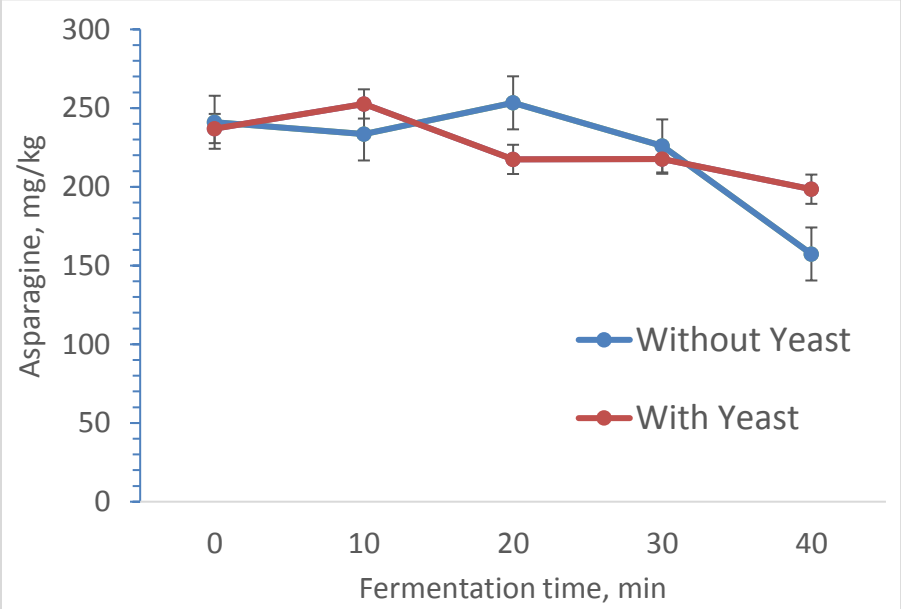

Figure 28 - The decrease of asparagine concentration during the fermentation

5.6 Conclusions

We found a decrease in asparagine concentration during longer extraction times, which suggested the presence of asparaginase activity in wheat. This assumption was further analyzed by measuring an amount of ammonia released

during incubation of wheat flour with water or buffer. Data on ammonia quantification showed an increase in ammonia over the course of the reaction. Higher evolution of ammonia when the reaction was run at pH 5 suggests that the enzyme may be active under normal food processing conditions. Finally, a reduction in asparagine during the fermentation of dough even without yeast further confirms the presence of asparaginase activity in flour. This is the first evidence of asparaginase activity in the wheat kernel, which can decrease concentration of acrylamide in baked products.

5.7 References

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6 Chapter 6. Application of NMR spectroscopy for detection acrylamide and characterization of compounds related to asparagine.

6.1 Abstract

This chapter will introduce our study on developing a method of acrylamide detection via NMR spectroscopy. Unfortunately, we could not measure acrylamide because its concentration in baked products is below the detection limits. However, a compound that had a similar appearance on NMR spectrum to acrylamide showed a strong positive correlation with asparagine. Further study of this compound may produce valuable information on asparagine accumulation and acrylamide formation potential during the Milliard reaction, which would lead to better understanding of conditions of acrylamide formation

6.2 Introduction

Acrylamide is a well-known neurotoxin and carcinogen that is widely found in thermally processed food [1,2]. Due to the enormous diversity of acrylamide containing products on the market regular screening of products available to the consumers is critically important [1,3]. Currently, methods for acrylamide detection require derivatization steps and skilled technicians which lead to high testing costs. For example, current standardized methods for acrylamide detection (LC-MS) require derivatization steps and skilled technicians which lead to high testing costs. Therefore, developing new methods of acrylamide

quantification which can be used as tool for providing reliable, high throughput method, with simple sample preparation is demanding area of research [4].

Application of new analytical tools for measuring acrylamide concentration in baked products is demanding area of research. Therefore application of NMR spectroscopy could be a valuable tool for acrylamide detection which does not require derivatization steps and skillful technicians. Moreover, NMR can be used for studying the correlation between asparagine with other components in grain, which is particularly important in terms of evaluation of potential acrylamide risk and better understanding conditions of acrylamide formation.

6.3 Materials and methods

6.3.1 Detection of pseudo acrylamide peaks

Sample (wheat flour or bread crust or crumb; 100 mg) was added to a 2 mL microfuge tube. Then 1 ml of deuterated chloroform (AcroSeal, NJ USA) was added and the test tube was vortexed for 5 s to disperse the sample. Extraction was carried out at 50 °C for 45 min with shaking on a reciprocal water bath at 200 rpm. Tubes were secured horizontally in the water bath. Following extraction, the samples were centrifuged at 5000*g* for 10 min at 4° C and then filtered through a 0.45 µm PTFE syringe filter (Merck Millipore, Cork Ireland). The filtration step precluded the formation of precipitates upon sample storage. The filtrate (0.4 mL) was transferred to a 5 mm NMR tube containing 60 µL of 0.15 mg/mL trimethylsilane (TMS) (AcroSeal, NJ USA) solution in deuterated chloroform as an internal standard.

6.3.2 Settings for NMR experiments

Proton spectra were acquired under automation using a 5 mm selective inverse probe. Collection of spectrum was performed by pulse sequence with a 90° pulse and a relaxation delay of 5 s. Each spectrum was acquired using 16 scans of 64,000 data points with a spectral width of 7309.99 Hz. Spectra were automatically Fourier transformed using an exponential window with a line broadening value of 0.5 Hz. Phasing and baseline correction were carried out manually within the instrument software. ^{1}H-NMR chemical shifts were referenced to tetramethylsilane TMS at δ=0.00. Concentration of acrylamide was calculated by integration of doublets at δ =6.4-6.45 and 5.8-5.85 and averaging the results of integration.

To confirm the doublets at δ= 6.3 and δ= 5.7 could be related to acrylamide, 2-dimensional homonuclear correlation spectroscopy (COSY) and heteronuclear single-quantum correlation spectroscopy (HSQC) were performed. The acquisition parameters for COSY and HSQC were: spectral width in F1 = 9090.909 Hz, F2 = 29165.938 Hz, size of real spectrum 1024, the number of scans=64. The spectral region of 5.5-6.5 ppm, inclusive, was suspended for further analysis.

6.3.3 Preparation of baked flour

Flour was baked in agreement with the procedure previously reported by Claus et al. [5]. 4 g of flour sample was weighed in open plate and heated at 170 °C for 20 min in the oven. Samples were collected from eight lots of commercial hard red winter wheat blends obtained from Bay State Milling (Quincy, MA USA).

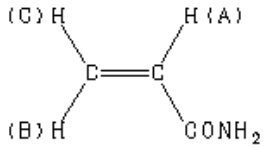
Whole kernels were milled using cyclone mill CT 193 Cyclotec (Foss, China). After cooling to room temperature, acrylamide content was determined as described previously.

6.4 Spiking of bread crust with solution of acrylamide

To validate determination of acrylamide 100 mg of bread crust was spiked with acrylamide solution (0.012 g/mL) in chloroform-d. Spiked bread was left exposed to air inside a fume hood for 48 h to evaporate the chloroform. Then bread crust was extracted and acrylamide concentration was determined as described.

6.5 Development of method for acrylamide detection by NMR spectroscopy

The first attempt of acrylamide detection was made by extraction of bread crust in D_2O:CH_3OD (80:20 v/v) in the same manner as described for ^{1}H-NMR determination of asparagine [6]. Data which available in literature (Fig. 29) shows that acrylamide signals appear in the range of 5.8 -6.32 ppm [7].



Assign.	Shift(ppm)
A	6.32
B	6.24
C	5.817

Figure 29 - Chemical shifts of asparagine

However, results which we collected were not applicable for quantification purposes. For the acrylamide standard, the trans protons on C-2 and C-3 had overlapping signals, and in a bread crust extract acrylamide resonances were weak, absent, or overlapping with other signals (Fig. 30).

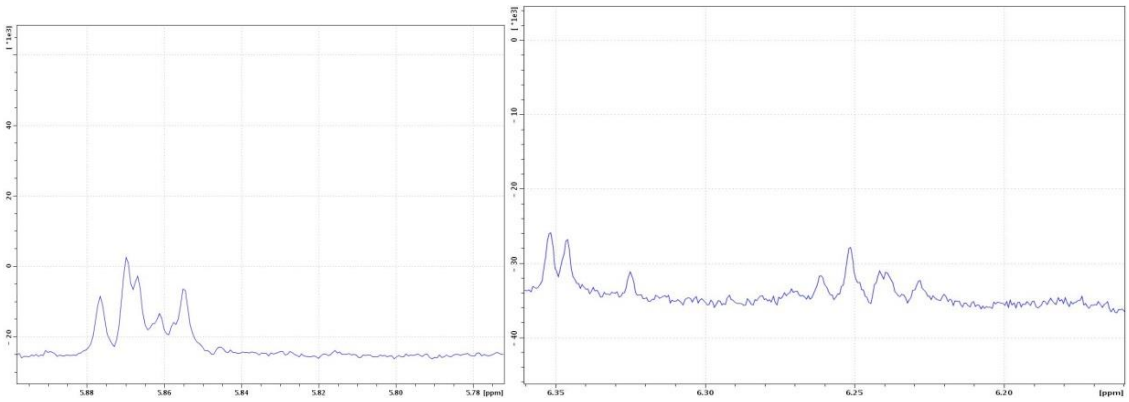


Figure 30 - Potential acrylamide signals collected in D2O:MeOD (80:20)

In contrast, extraction of bread crust in chloroform-d resulted in clear doublets at δ=5.7 and δ=6.3 with same chemical shifts as pure acrylamide (Fig. 31). These doublets corresponded to 3-H_b and 3-H_c on the acrylamide spectrum. The other C-linked proton in acrylamide (2-H_a) showed a doublet of doublets at δ= 6.2, but this signal was obscured in the bread extract, probably due to overlapping signals with other compounds in the extract. However, we anticipated that the doublets at δ=5.7 and δ=6.3 were clear and could be used for quantification.

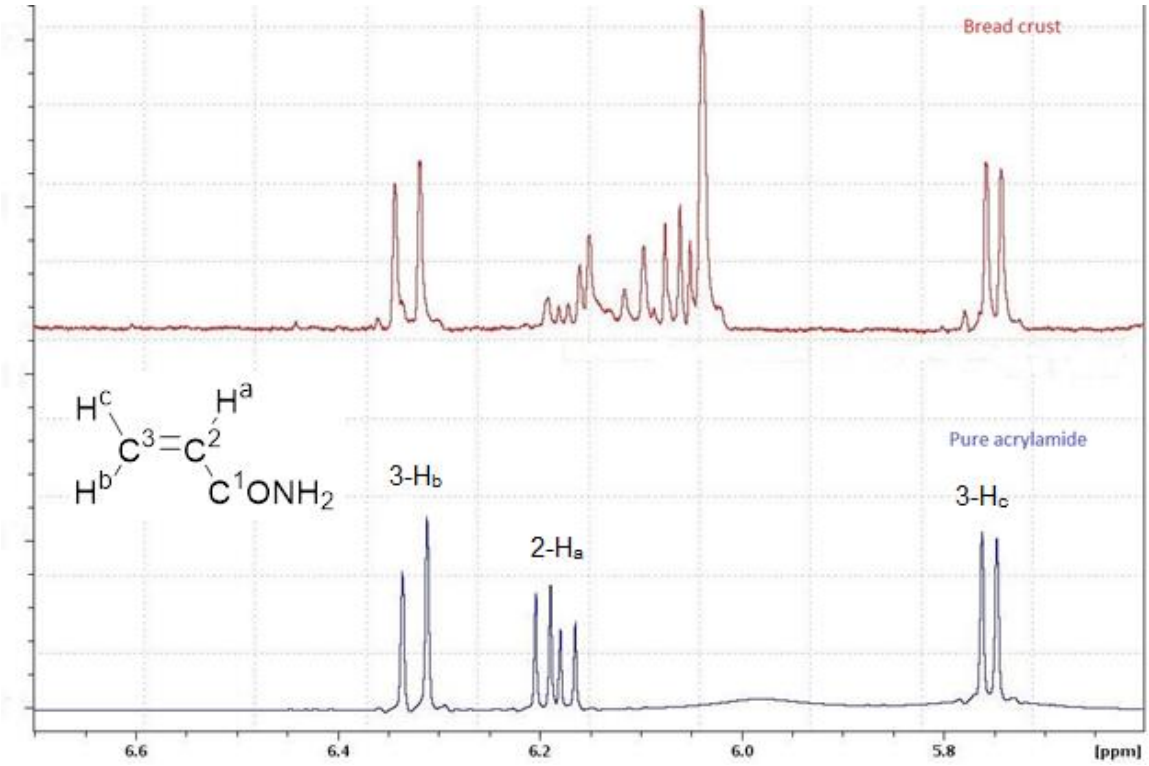


Figure 31 - 1H-NMR spectrum (δ=5.7-6.7) of bread crust extract (top) and acrylamide (bottom) in chloroform-d; the structure of acrylamide is shown in the inset

To confirm that these doublets corresponded to acrylamide, 2-dimensional NMR spectroscopy was employed. COSY showed strong coupling between both of these signals and those at δ= 6.2 (Fig. 32), indicating that the protons 3-H_c and 3-H_b were attached to the same carbon and located 3 bonds away from proton(s) resonating at δ= 6.2. This was the location of 2-H^a in the pure acrylamide spectrum. Furthermore, a weak coupling between 3-H^b and 3-H^c was also

detected since they were situated on the same carbon (2-bonds away).

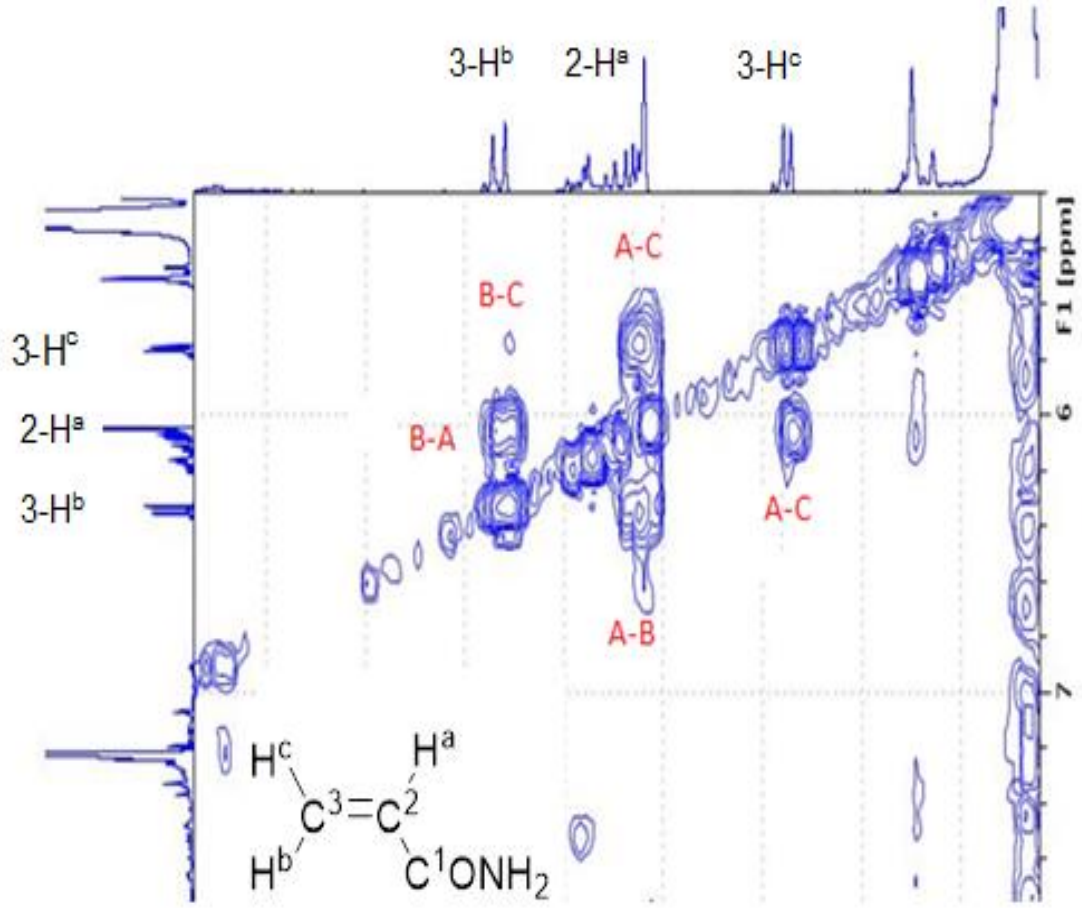


Figure 32 - Two-dimensional COSY spectrum of bread crust extract NMR

HSQC showed coupling between the carbon at δ=130 and the protons at δ= 5.7 and δ= 6.3 (Fig. 33). This result is consistent with the coupling between C-3 and 3-H_a and 3-H_b on acrylamide. C-H coupling was also apparent between carbon at δ=128 and a proton at δ=6.2, which would correspond to the location of 2-H_a, but was obscured by another compound in the extract.

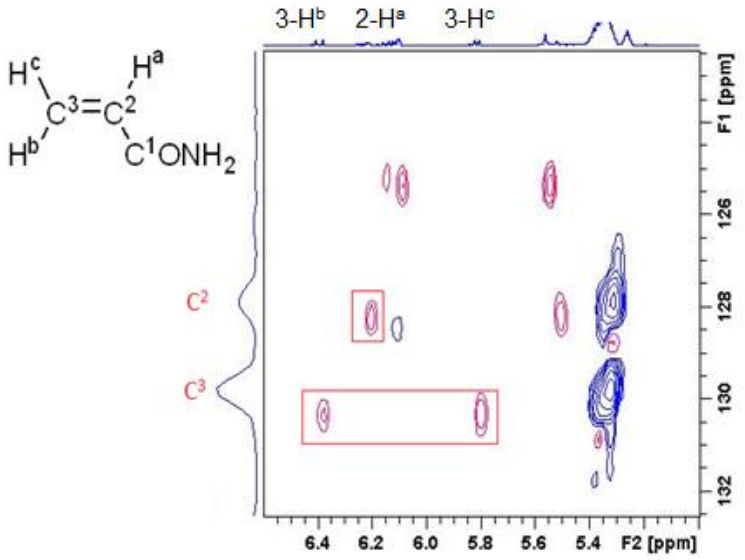


Figure 33- Two-dimensional HSQC spectrum of bread crust extract NMR

These observations were consistent with the structure of acrylamide. Thus, the doublets at δ= 5.7 and δ=6.3 were used for quantification.

However previously detected peaks was also present in unheated flour (Fig. 34), which give evidence to believe that they do not relate to acrylamide. Moreover concentration of this compound is thousand times higher compare to data reported in the literature on acrylamide.

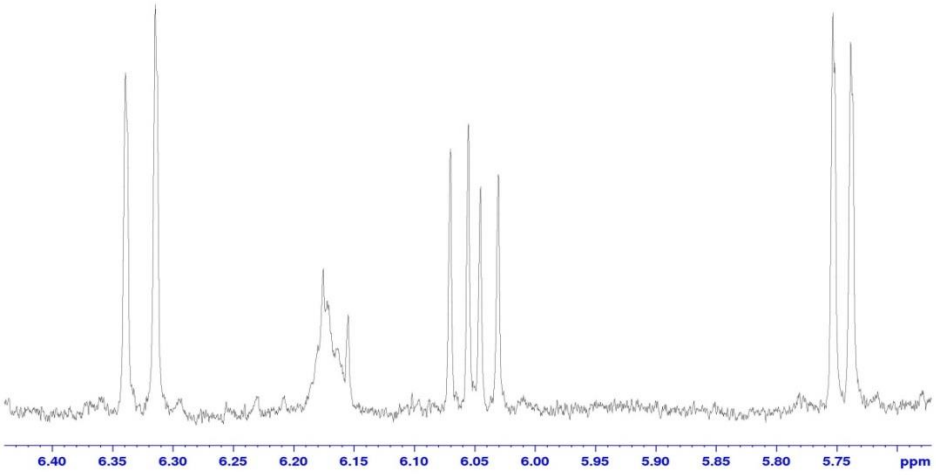


Figure 34. Unheated wheat flour

In addition to that spiking of bread crust with the solution of acrylamide showed additional appearance of peaks on the spectrum which can be related to acrylamide spike. Peaks placed in red bars (Fig.35) are shifted from peaks of pure acrylamide (Fig.31) which can be due to pH of bread crust or reaction between acrylamide and other components of bread crust extract.

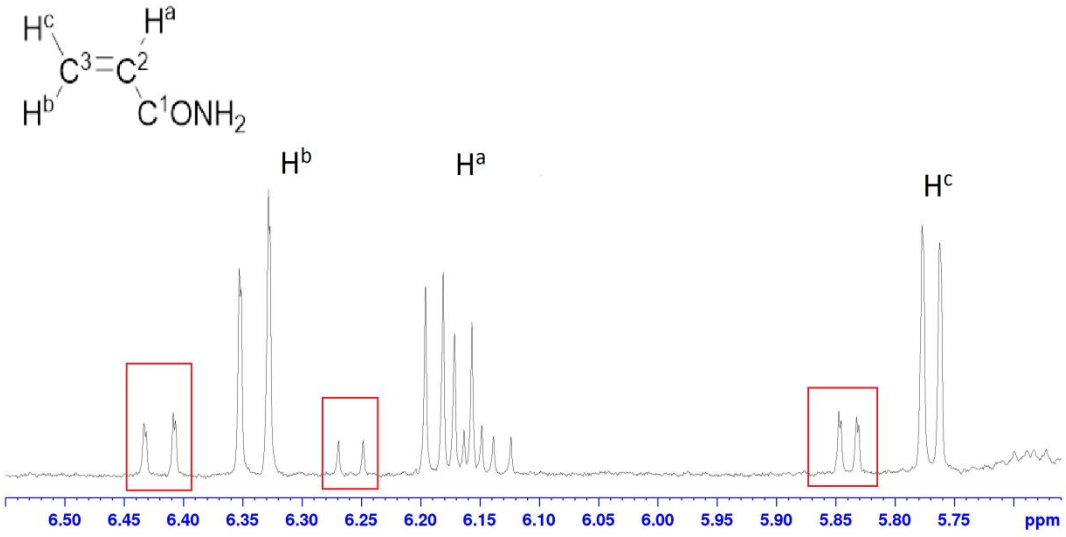


Figure 35 – An appearance of new peaks after spiking

6.6 Study of correlation between asparagine level and unknown compound

The relationship between asparagine concentration in milled whole wheat and pseudo acrylamide (peaks at 5.7 and 6.3) in heated milled whole wheat was determined (Fig. 36).

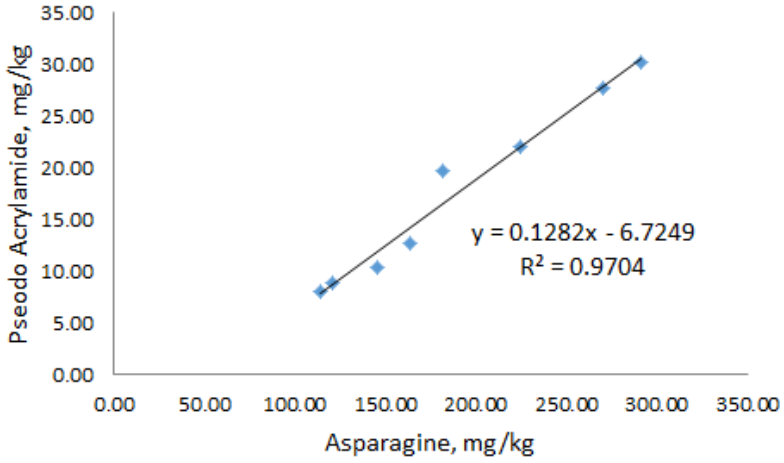


Figure 36 - The relationship between asparagine concentration in milled whole wheat and unknown peaks in heating the milled sample

As we can see asparagine level strongly correlates with the concentration of unknown compound extracted in nonpolar chloroform solvent. This gives a clue that our unknown compound is lipophilic in nature (signals represent proton of an unsaturated fatty chain R, with situated closely to electron withdrawing group). Therefore we purpose that detected peaks represent unsaturated protons of of steryl ferulates (Fig. 37), which is major lipophilic antioxidants in wheat [6]. However more experiments are needed to define the exact structure of unknown compound.

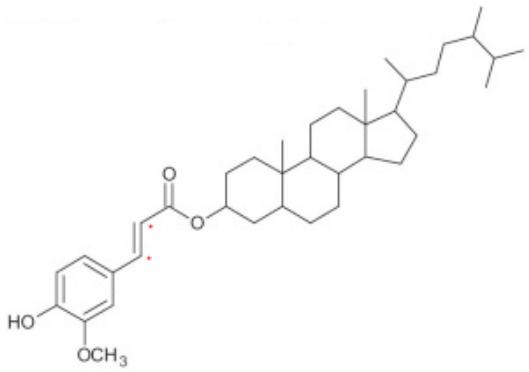


Figure 37 - Structure of steryl ferulates (campestanyl ferulate)

6.7 Conclusions

Based on data provided in this chapter we can conclude that acrylamide cannot be quantified by extraction in deuterated chloroform. However, compound which has similar peaks patterns as acrylamide on proton NMR spectrum showed significant correlation with asparagine. Therefore further characterization of unknown compound which showed significant correlation with asparagine level can lead to better understanding of the mechanism of acrylamide formation.

6.8 References

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7 General conclusions

This thesis introduced a first look into the variation in asparagine level in Nebraska wheat in the context of risk of acrylamide accumulation in baked products. Location was found to have a significant effect on asparagine concentration, with the higher level of asparagine possibly explained by a longer period between flowering and harvest and influence of sulfur deficient soil. Furthermore, significant positive correlations between asparagine concentration and kernel diameter and size.

Along the same lines, evaluation of asparagine concentration in proso millet was studied. It was shown that the average concentration of asparagine in proso millet was almost three times lower compared to the average for wheat sample from our previous study. Moreover the level of asparagine in proso was not influenced by additional artificial irrigation, which gives evidence to hypothesize that drought stress will not significantly increase the concentration of asparagine, however, further studies are needed in this area.

Chapter 4 of my thesis showed the potential presence of asparaginase in wheat kernels, which was not previously reported in the literature. I pursued this idea after observing that asparagine concentration decreased during longer extraction times. The presence of asparaginase was confirmed by measuring both the decrease in asparagine and the increase in ammonia during incubation of flour-water slurries. I also showed that asparagine level in dough decreased without the addition of yeast. Based on different rates of ammonia accumulation

between different cultivars and higher enzyme activity at pH 5 compared with pH 8 suggested that it is present in the wheat kernel itself and does not relate to microbial activity.

Finally, our attempt to determine acrylamide in deuterated chloroform did not show any potential application for acrylamide quantification. However, we discovered correlation of asparagine with the compound which can be unsaturated fat or phenol containing amino acid which provides peak in the downfield region of ^{1}H NMR spectrum in d-chloroform. This correlation can be useful for better understanding of the mechanism of acrylamide formation.

8 Appendix

In my future work I will focused my efforts in analysis of ^{1}H NMR spectrum of flour in principal component analysis (PCA). By the means of PCA we would like to see if spectrum of freshly milled wheat in deteurated chloroform (which include numerous peaks from lipids components Fig. 38) will be significantly different from flour which had been stored for a long time. In addition to that our preliminary data pointing out on significant differences in ^{1}H NMR spectrums between different wheat cultivars (Fig. 39).

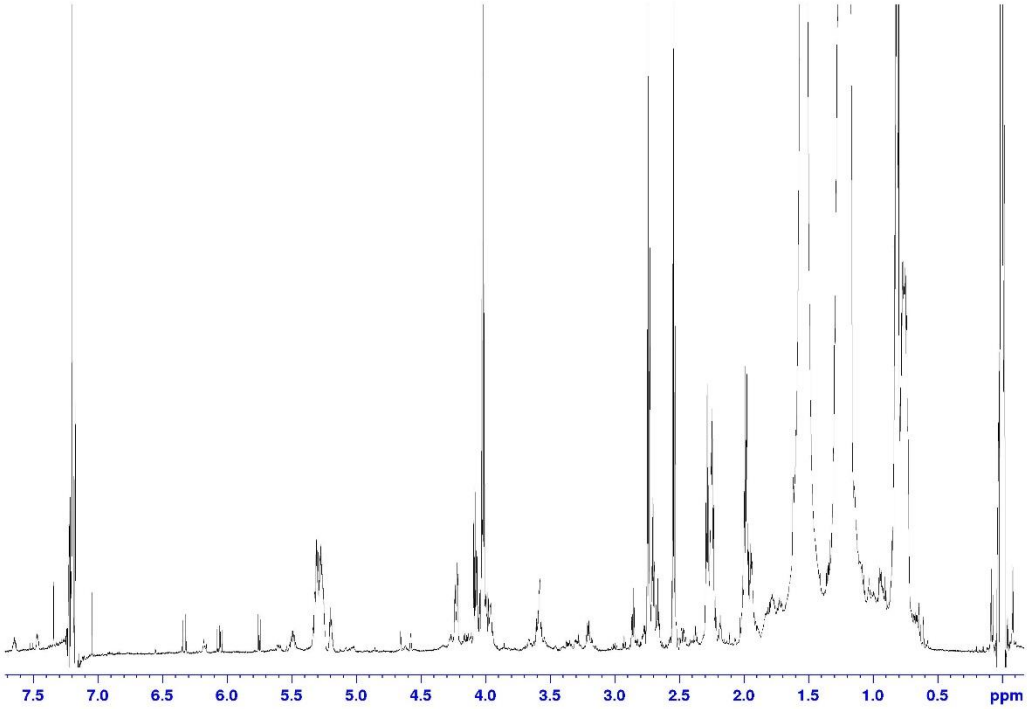

Figure 38 -Spectrum of freshly milled wheat

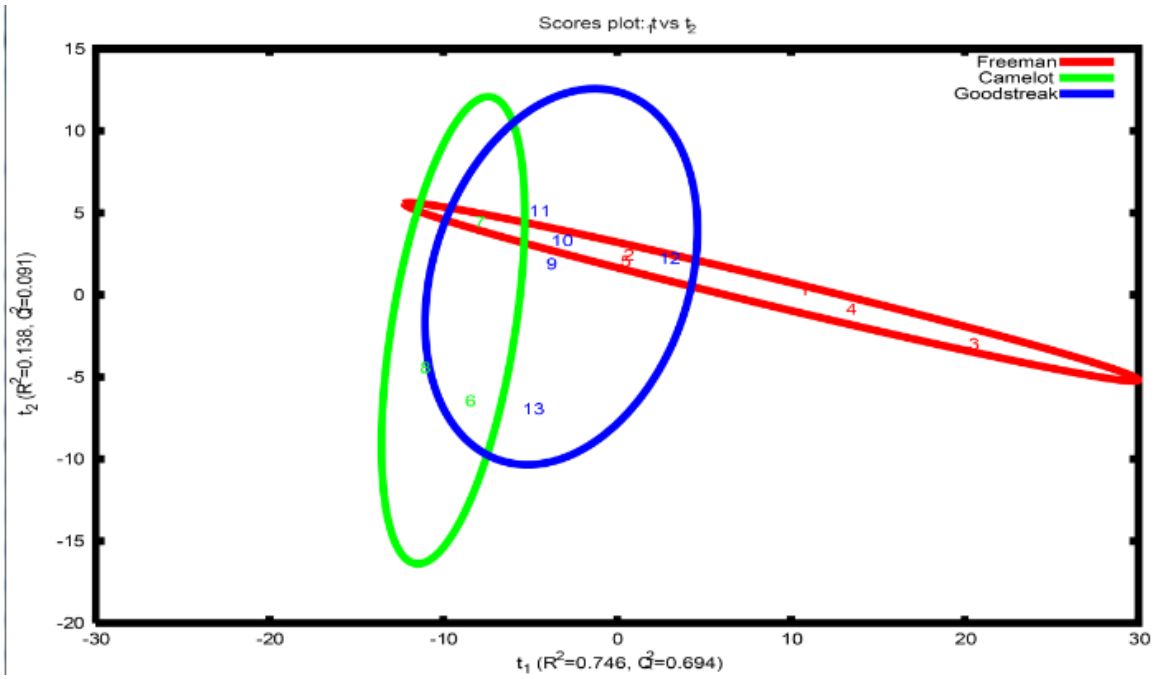

Figure 39 - PCA of different wheat cultivars