DEVELOPMENT AND OPTIMIZATION OF A MICROWAVE-ASSISTED PROTEIN HYDROLYSIS METHOD TO PERMIT AMINO ACID PROFILING OF CULTIVATED AND WILD WHEATS AND TO RELATE THE AMINO ACID TO GRAIN MINERAL CONCENTRATIONS

By
KHALED QABAHA

Submitted to Graduate School of Engineering and Natural Sciences
In Partial Fulfillment of
The Requirements for the Degree of
Philosophy of Doctorate

Sabanci University
May 2010

DEVELOPMENT AND OPTIMIZATION OF A MICROWAVE-ASSISTED PROTEIN HYDROLYSIS METHOD TO PERMIT AMINO ACID PROFILING OF CULTIVATED AND WILD WHEATS AND TO RELATE THE AMINO ACID TO GRAIN MINERAL CONCENTRATIONS

APPROVED BY:

Assoc. Prof. Levent Öztürk (Thesis supervisor)……

Prof. İsmail Çakmak …….

Assist. Prof. Alpay Taralp……..……..

Assist. Prof. Mehmet Serkan Apaydın ……...……..

Prof. İsmail Türkan……...

DATE OF APPROVAL: 13.05.2010

Acknowledgements

I would like to express my sincere appreciation and heartily thankfulness to my supervisor, Dr. Levent Öztürk, for his encouragement, guidance, patience and support from the formative stages to the final level of this work. I owe him an immense debt of gratitude for his kindness, patience, time and insight throughout the research.

I owe Dr. Alpay Taralp great gratitude for his help, support and encouragement.

My extended special thanks to Prof. İsmail Çakmak, Prof. İsmail Türkan, Dr. Mehmet Serkan Apaydin and Dr. Hikmet Budak for their help and valuable comments.

My sincere thanks are extended to Dr. Thaer Abdelghani, Dr. Suliman Abadi and Dr. Jawad Abadi for their moral support and help.

I owe my deepest gratitude to my parents because of their love, encouragement and advice. My sincere love and thanks goes to my wife, Inam, whom without her encouragement and understanding this work has not been possible. I have a great deal of thanks to my children: Ala, Hana, Amal, Mohammad, Ibrahim and Abdullah for their patience and support. I also owe special gratitude for my Brother Mahmood amd my sisters Amal and Samar.

I am in debt to the team members, Gamze Altıntaş, M. Atilla Yazici and Veli Bayır for their team spirit work, help, and kindness.

Thanks also go to my friends Yaser Alkahloot, Belal Amro, Ahmed Abdalal, Momin Abu Ghazala, Samir Sadiq, Ahmad Al-gharib, Iyad Alhashlamoon and Amer Fayez for their help and support by making my life easier and joyful.

For the last but not least I want to acknowledge the financial support provided by the Erasmus Mundus University-ECW.

ABSTRACT

DEVELOPMENT AND OPTIMIZATION OF A MICROWAVE-ASSISTED PROTEIN HYDROLYSIS METHOD TO PERMIT AMINO ACID PROFILING OF CULTIVATED AND WILD WHEATS AND TO RELATE THE AMINO ACID TO GRAIN MINERAL CONCENTRATIONS

Khaled Ibrahim Qabaha
Biological Sciences and Bioengineering
PhD Thesis, 2010

Thesis Supervisor: Assoc. Prof. Levent Öztürk

Key Words: microwave-assisted protein hydrolysis, amino acid profiling, wheat, mineral nutrients

Wholegrain flour from durum wheat (*T.durum*, cv. Balcali-2000) was subjected to amino acid analysis following microwave-assisted acid hydrolysis. To optimize this new method, a range of sample masses (100-500 mg), incubation temperatures (130-170^{o}C) and time intervals (1-4h) were assessed. Overall, the greatest recovery of amino acids was obtained when 200 mg of wheat flour sample was hydrolyzed at 150^{o}C for 3 h. The developed microwave hydrolysis method was confirmed to yield comparable findings with classic reflux methods. Integration of all amino acid signals corresponded to 85 % of the total protein content calculated by total N. The highest signal reflected the combined contributions of glutamic acid and glutamine, in accord with previous findings. Also as expected, proline was found to rank in second place. It follows to reason that an optimized microwave-assisted hydrolysis method may describe a rapid means to compare the constitution of different genotypes of wheats and may further show merit and general applicability towards the rapid analysis of commercially important crops and their end-products.

In all wheat species and genotypes Glu was the most abundant amino acid, followed by Pro, whereas Met sln, Lys and Thr were the most limited. The quantities and ratios of individual amino acids were consistent with the literature data and the quantitative order of major and minor amino acids did not change in genotypes or species. However, amino acids exhibited significantly high variations among genotypes and species which can be exploited to enhance specific and/or total amino acids (i.e. protein) in high yielding cultivated wheats through selection, breeding and targeted molecular approaches. Although the existence of significant associations between a few amino acids and mineral nutrients, it was not possible to define or explain a co-transport or co-accumulation mechanism. Future research should focus on the phloem transport and mobility of metal binding proteins and organic ligands, rather than individual amino acids. A major finding of this study was the augmentation of correlations (among amino acids, nutrients and amino acids with nutrients) upon prescreening for contrasting grain N (or protein) concentration. Advancements in increasing the grain protein content of wheat can significantly contribute to enrichment of grains with almost all mineral nutrients except K and Ca.

ÖZET

TARIMI YAPILAN VE YABANİ BUĞDAYLARDA AMİNO ASİT PROFİLLEMESİ İÇİN MIKRODALGA-YARDIMLI HİDROLİZ METODU GELİŞTİRİLMESİ VE OPTİMİZASYONU VE AMİNO ASİTLERİN TANE MİNERAL KONSANTRASYONU İLE İLİŞKİLERİNİN BELİRLENMESİ

Khaled Ibrahim Qabaha
Biyoloji Bilimleri ve Biyomühendislik
Doktora Tezi, 2010

Tez Danışmanı: Doç. Dr. Levent Öztürk

Anahtar Kelimeler: mikrodalga-yardımlı protein hidrolizi, amino asit profillemesi, buğday, mineral besinler

Durum buğdayından (*T. durum*, Balcalı 2000) elde edilen tam buğday unu mikrodalga-yardımlı asit hidroliz sonrasında amino asit analizine tabi tutulmuştur. Bu yeni metodun optimizasyonu için farklı numune ağırlığı (100-500 mg), inkübasyon sıcaklığı (130-170^{o}C) ve süresi (1-4 h) irdelenmiştir. Sonuç olarak amino asitlerin en yüksek geri kazanımı 200 mg buğday unu numunesinin 150^{o}C'de 3 saat hidrolizinde elde edilmiştir. Geliştirilen mikrodalga hidroliz yöntemi klasik reflü yöntemine benzer sonuçlar verdiği teyit edilmiştir. Elde edilen amino asitlerin toplamı, toplam N ile hesaplanan protein kapsamının % 85'ine karşılık gelmiştir. Önceki çalışmalarla benzer olarak glutamic asit ve glutamin toplamı en yüksek değere sahip olmuştur. Yine beklendiği üzere prolin de ikinci sırada yer almıştır. Sonuçlar optimize edilmiş mikrodalga-yardımlı hidroliz metodu ile farklı buğday genotiplerinin amino asit kapsamlarının hızlı şekilde karşılaştırılabileceğini ve metodun diğer tahıl türlerinde ve bunlardan üretilen ürünler için de kullanılabileceğine işaret etmektedir.

Tüm buğday türlerinde en fazla miktarda bulunan amino asit Glu olarak bulunurken bunu Pro takip etmiş, Met sln, Lys ve Thr ise en düşük değerleri almıştır. Amino asitlerin bireysel miktar ve oranları literatür verileri ile uyumlu bulunmuş, majör ve minör amino asitlerin miktarsal sıralaması tür ve genotipler arasında değişim göstermemiştir. Buna karşın, tür ve genotiplerin amino asit konsantrasyonları arasında, bireysel ve/veya toplam amino asitlerin (proteinin) yüksek verim kapasiteli çeşitlerde seleksiyon, ıslah ve hedeflenmiş moleküler yöntemlerle arttırılmasına olanak sağlayacak düzeyde önemli varyasyon olduğu gösterilmiştir. Bazı amino asitler ve mineral besin elementleri arasında önemli ilişkiler bulunmasına karşın, bunların birlikte taşınması ve biriktirilmesine dair mir mekanizmanın tanımı veya açıklaması mümkün olmamıştır. Gelecekte yürütülecek çalışmalar bireysel amino asitlerden çok, metal bağlayan protein ve organik ligandların floem taşınımı ve mobilitesi üzerine odaklanmalıdır. Bu çalışmanın ortaya koyduğu önemli bulgulardan biri ön eleme ile tane N konsantrasyonu bakımından farklı genotiplerin seçilmesi sonucunda korelasyonlarda gözlenen artışlar (amino asitler arasında, besin elementleri arasında ve amino asitlerle besin elementleri arasında) olmuştur. Buğdayda tane protein kapsamının arttırılmasına yönelik çalışmalar, K ve Ca dışında tüm mineral besinlerin tanede zenginleşmesine katkı yapabilir.

TABLE OF CONTENTS

List of Figures

List of Tables

List of Abbreviation

g: Gram

h: Hour

kg: Kilogram

L: Liter

M: Meter

μm: Micrometer

mg: Milligram

min: Minute

mmole: Millimole

wf: Wheat flour

r: Correlation coefficient

1 INTRODUCTION

1.1 Relevance of Proteins to Life

Protein is an important key stone in body functions such as formation of antibodies, wound repair, protein synthesis (Suryawan, et al., 2009), modulation of gene expression (Palis, et al., 2009), intestinal integrity (Wang, et al., 2009) and regulation of cellular signaling pathways (Rhoads, et al., 2009). Protein, together with micronutrient malnutrition is predominant in developing countries where cereals are the main source for protein intake while meat, rich in protein, iron and other vital micronutrients, is the main source in the developed countries (FAO, 2009; Ranum, 2001).

Cereals have the biggest share among the dietary components in total energy consumption of the whole world with an average of 47% and in many countries such as Bangladesh strikes to more than 70%. Among the cereals, wheat is consumed the most which provides a huge proportion in the nutrition of both human and livestock (FAO, 2009; Shewry, 2009).

Green revolution was successful in decreasing the hunger of the world`s poor. This success has kept cereal as the most available and cheap source of energy and protein, but it has also reduced the diversity of food intake especially in the developing countries (Welch, et al., 1999; Demment, et al., 2003). Cereal production exceeded 2100 million tons in the year of 2005. Maize, wheat and rice accounted for 85% of the total cereal production. USDA World Wheat Collection screening showed that the protein content varied from 7% to 22% in different wheat lines. Third of this variation is due to genetic factors and two-thirds are due to non-genetic factors involving mainly environmental conditions (Vogel, et al., 1978). Many mutagenesis and conventional breeding attempts were carried out to increase the wheat protein content, such as the selection studies performed at CIMMYT which resulted in opaque-2 lines with high concentration of lysine (Shewry, 2007; Prasnna, et al., 2001; Gibbon, et al., 2005). Wheat grain includes all essential amino acids including Histidine, Isoleucine, Leucine, Lysine, Cysteine, Methionine, Phenylalanine, Tryptophan, Tyrosine, Threonine, and Valine which human body needs but can not synthesize (Moose, 1990; Tamis, et al., 2009). However, the content of lysine, threonine, and sulfur containing amino acids (cysteine and methionine) is low in wheat compared to food from animal origin (Elango, et

al., 2009). This is very important for children, who need more essential amino acids than adults for their development and growth, and also for people in the developing countries that rely more on cereals and particularly wheat for their protein and calorie intake (Tamis, et al., 2009).

Reproducible and quick separation and determination of amino acids, after hydrolyzing the peptide bonds that joins the amino acids together, helps in identifying, quantifying and characterizing the protein. In the case of wheat protein, the current method of choice is based on the traditional reflux method, which requires approximately 24 hours to achieve analysis-quality hydrolysates (Basak, et al., 1993; Weiss, et al., 1998). Rapid hydrolysis of proteins coupled with high recovery rate of all of the amino acids offers a powerful tool in protein research, nutritional, and biochemical investigations.

Many important functions are carried out by proteins such as the transport of molecules in body fluid. Movement of the cells and the whole organism depends on muscles which are in fact contractile proteins. Most of the biochemical reactions are catalyzed by enzymes that are consistent of large and complex protein molecules. Likewise, signaling molecules, hormone receptors and transcription factors that switch the genes on and off are also proteins (Kimball, 2009; Rhoads, et al., 2009). Antibodies that have an important physiological role in defending both the plant and animal tissue from pathogens are proteins. Conjugated proteins, as those combined with chlorophyll and nucleic acids, have an important role in photosynthesis and gene replication (Spurway, 2008). From these examples listed, it is obvious that the normal functioning of a given organism is totally dependent on the synthesis and availability of free amino acids, which are the building blocks of proteins.

1.2 Wheat Grain Proteins

A mature wheat grain contains nutrients and products of biosynthesis accumulated over the grain's life time. Proteins and carbohydrates are synthesized from water and nutrients that were taken up from the soil by the root and shoot, and the carbon taken up from the atmosphere (Spurway, 2008).

The mature wheat grain is composed mainly of high starch content, around 72% of the total dry weight present only the endosperm, and the protein content is between 6-16% and distributed all over the grain (Shewry, 2007).

Wheat flour contains more than thousand proteins that can be detected by 2-D gel electrophoresis. But many have minimal importance in the quality of the bread. The major wheat flour protein types are albumins, globulins, gliadin monomers and the low and high molecular mass glutenin subunits (Wang, et al., 2007).

Also, wheat grain proteins differ in solubility, albumin is water soluble, globulins are insoluble in water but soluble in salt solutions, moreover, gliadins are soluble in 70-90% of ethanol, and glutenins are insoluble in saline or neutral aqueous solutions but soluble in alcohol as monomers, dimmers, or even small polymers. Glutenins are present in flour as insoluble, large polymeric aggregates that surround the granules of the starch having the highest effect on the bread making quality of flour (Osborne, 1907; Dupont, et al., 2005).

The globulins and albumins are the cytoplasmic and metabolically active proteins, but glutinins and gliadins are mostly storage proteins. The metabolically active proteins are present in the germ and pericarp-aleurone layers, but the storage proteins are found in endosperm (Lasztity, 1996). There are major differences between the storage and the cytoplasmic proteins and their amino acid compositions. Large proportion of the storage proteins is glutamic acid and proline and a small proportion of arginine, lysine, tryptophan, and threonine. The metabolically active proteins contain much less glutamic acid and proline, and higher proportions of arginine and lysine which allow these proteins to have higher nutritive value, and less functional properties (Spurway, 2008).

Proteins that are metabolically active, mainly globulins and albumins, are formed in initial stages of kernel development. This is associated with the early development of the embryo and the aleurone layer, and makes the aleurone layer be separated from the outer layer of endosperm cells at about 12-14 days after synthesis. These proteins, in total, make up less than 20% of the fully made kernel. Storage proteins appear first in the developing endosperm around 10 days after synthesis and kept synthesized until the kernel become mature (Buttrose, 1963; Jenings, et al., 1963; Simmonds, 1978).

Most of the wheat utilized by humans is processed from the white flour, as a result of milling to remove the germ (embryo) and the bran (testa, pericarp, nuclear layer, and aleurone layer). The flour is mainly consisted of the endosperm which contains a high proportion of starch and gluten proteins. The gluten proteins make a continuous matrix in the cells of the mature dry endosperm. When the water is added to the flour to form dough, the protein matrices in the endosperm cells are brought together to a continuous network. This provides the visco-elastic property of the dough and the expansion characteristic during fermentation and baking into bread or processed into noodles and pasta. The strong dough (highly visco-elastic) contains large amounts of high molecular mass polymers of glutenins (Shewry, et al., 2002; Field, et al., 1983). Payne et al have demonstrated that allelic variations in the structure of the high molecular weight (HMW) prolamins (HMW subunits of glutenin) was highly correlated with differences in bread making quality of European bread wheat (Payne, 1987).

Human body can synthesize most of the essential amino acids except arginine (important for the young but not for the adults), histidine, leucine, isoleucine, lysine, methionine, threonine, phenylalanine, valine, and tryptophan. The essential amino acids are supplied by foods, mainly by cereals and particularly by wheat (Ozman, et al., 2009). High protein content is generally accepted as the primary quality parameter and the main guideline for wheat trade transactions. Glutamine and proline constitute almost half of the wheat grain proteins, but the other amino acids which are considered essential for the human diet are considerably low such as lysine, tryptophan, methionine, isoleucine and threonine (Acquistucci, et al., 1995).

Amino acid composition and protein content in the wheat grain depends mainly on the genotype and characteristics of the environment, such as nitrogen-application time, nitrogen-fertilization rate, nitrogen concentration in the soil, availability of soil- moisture and temperature through grain-filling (Luis, et al., 2007).

In order to estimate the protein content in cereal grains, the classical approach is to analyze the total nitrogen (N) concentration and convert this to protein by multiplying with a nitrogen-to-protein conversion factor. When the whole N is assumed as protein-bound, the conversion factor is 6.25 based on the estimation that their proteins contain approximately 16% N. For wheat usually 5.83 is used for N-to-protein conversion (Merrill AL, Watt BK. 1973) although some studies claim that even 5.83 is still high. Due to differences in amino acid composition and the presence of non-protein compounds that contain N, the use of a

specific conversion factor will introduce significant errors. Examples of compounds that contain N beside proteins are ammonia, urea, nucleic acids, nitrates, vitamins, phospholipids, alkaloids, and nitrogenous glycosides (Dupont, et al., 2005; Fujihara, et al., 2008).

According to the World Health Organization, around 160 million children under five years of age lack adequate protein intake leading to health and economical problems for the societies. The two main types of wheat, hexaploid wheats (used primarily for bread) and the tetraploid wheats (used primarily for pasta) almost account for 20% of all calories utilized worldwide (Uauy, et al., 2006). On a yearly basis world wheat production is about 620 million tons providing about 62 million tons of protein. It has been claimed that only a little progress could be achieved in increasing wheat protein, Zn and Fe content due to environmental and genetic factors (Uauy, et al., 2006).

1.3 Zinc and Iron and Their Relevance to Life

Zinc and Fe are essential nutrients for maintaining the normal functioning of the human body. Many studies have indicated that almost three billion people are affected by Fe deficiency (Welch, et al., 1999), and almost one third of the population of the developing countries may have Zn deficiency. When both deficiencies are considered almost half of the world's population is thought to be affected (Hotz, et al., 2004). Both Zn and Fe deficiencies may cause severe health troubles such as growth retardation, impairments in mental development and high susceptibility to infectious diseases among children, also defects in the immune system, cognitive and mental development, physical growth, iron deficiency anemia and increase in both mortality and morbidity (Black, 2003; Walker, et al., 2009; Ozturk, et al., 2006). Beside health effects, micronutrient deficiencies may also be associated with decreased work productivity and reduced national income especially in developing countries (Bouis, 2003).

Zinc deficiency in soils and plants occurs worldwide; about 50% of the soil samples from 25 different countries are proved to be low in Zn concentration. Turkey is one of those countries with almost 14 million hectares of cultivated land have shown to be Zn deficient, which in turn leads to decrease in the yield of wheat grain. In one hand, Zn deficiency decreases the nutritional quality of the grain, and in the other hand it decreases the cereals resistance to diseases and affects the nutritional quality of the grain (Cakmak, et al., 1999).

It has been found that Zn is an important mineral in maintaining and enhancing mammalian immunity. For a long time, it was well known that Zn was essential for both animals and plants, but about 40 years ago it was also found to be essential for human health (Prasad, 2008).

In general, wild and less-advanced wheat species were used to improve the quality of the modern wheat. They were utilized as a source for genes to enhance the modern wheat quality. As an example; the A-genome in primitive and wild diploid wheat has been used to increase the disease resistance of cultivated wheats (Kerber, et al., 1973; Valkoun, et al., 1986; Hussien, et al., 1997). Also, the D genome absence in tetraploid wheat may explain why it is low in Zn. The genes responsible for the Zn expression most likely locate on many chromosomes of the D genome. That is why transfer of the whole genome from *Aegilops tauschii* (source of the D genome of hexaploid wheat) improves the growth of tetraploid wheat under Zn deficient, but not under Zn sufficient conditions (Cakmak, et al., 1999).

Human health in many countries especially in the developing ones is also affected by micronutrient deficiencies. About 50% of the 6-month old children, 50% of women at their reproductive age, and 30% of children at their school age have iron deficiency anemia (Initiative, 2009).

According to Cakmak et al, an important reason for the widespread of the micronutrient deficiencies is the high intake of diet with little diversity usually containing one or two staple foods. In the developing countries due to poverty, many people rely on cereal-based food to obtain their energy and protein, and the animal based food with high amounts of micronutrient is not common (Cakmak, et al., 2004). In less developed countries, wheat, rice, and maize are the main staple food in the diet and about 60% of the daily calorie intake is supplied by wheat. Therefore; an increase in Fe and Zn concentrations in the wheat seeds will decrease their deficiencies in humans and animals that are dependent to wheat as the staple source of food and energy. Besides that wheat, rice, and maize contain low Fe and Zn, they are also rich in compounds that limit the bioavailability of these micronutrients such as high fiber and phytate (Frossard, et al., 2000; Welch, et al., 2004).

One way of correcting the micronutrient malnutrition problem in populations is suggested to be by food fortification and supplementation with the vital micronutrients, however it is an expensive way and hard to apply especially in the developing countries, and in particular, the rural areas (Bouis, et al., 2000; Bouis, 2003). Instead; traditional plant breeding and genetic engineering methods are being used to enrich the cereals with Fe and Zn which is considered as more cost-effective and sustainable (Frossard, et al., 2000; Cakmak, et al., 2002; Welch, et al., 2004).

There are many factors that play a role in increasing the micronutrient concentration in cereal grains. The genetic variations for Fe and Zn among cereal species and genotypes are a major factor. Other factors are related with the environment and may have more impact than the genetic variations, such as fertilizer management, water availability and soil properties. There are preliminary studies that indicated both wild and primitive wheats (as *Triticum monococum*, *Triticum dicoccon*, and *Triticum dicoccoides*) may be good genetic donors for enhancement of micronutrients in the cultivated wheats. *Triticum dicoccoides*, a wild wheat germplasm, have shown the highest concentration and the largest variation of micronutrients particularly for Zn, and represents as a very good donor to increase the concentrations of Fe and Zn in cultivated wheat (Cakmak, et al., 2000; Cakmak, et al., 2004).

1.3.1 Role of zinc in the function of human immune system

Effect of Zn on health has been studied in the last four decades. Zinc deficiency in humans could be mild moderate or severe, affecting immunological, biochemical, and clinical functions. Severe Zn deficiency has been found in patients with enteropathica (a genetic disorder), acrodermatitis, excess alcohol intake and penicillamine therapy. The signs and symptoms of severe zinc deficiency in humans are various including diarrhea, dermatitis, emotional disorders, weight loss, intercurrent infections because of cell-mediated immune dysfunctions, neurosensory disorders, delay in healing of ulcers and hypogonadism in males. The conditions may become fatal in untreated patients. Moderate zinc deficiency symptoms include hypogonadism in adolescents, growth retardation, rough skin, mental lethargy, poor appetite and cell mediated immune dysfunctions. In mild zinc deficiency, signs and symptoms include oligospermia, decreased serum testosterone level, decreased interlukin-2 activity, decreased natural killer cell activity, decreased thymulin activity, decreased dark adaptation, hyperammonemia, and decreased lean body mass (Prasad, et al., 1988; Beck, et

al., 1997). Thymulin is produced from the thymus and it plays a role in T-cell activity and requires zinc for its function. It binds with T cell receptors promoting its functions as production of interlukin-2, cytotoxicity and suppressor capability (Prasad, et al., 1988). There are studies that estimated almost 2000 transcription factors are affected by zinc (Prasad, et al., 2001).

Zinc deficiency is favored in people with high cereal protein consumption due to excess phytate content in cereal based foods. Phytate is defined as an anti-nutrient which prevents absorption and thus bio-availability of Fe and Zn (Cakmak et al 2010).
A sufficient level of Zn is required to inhibit the plasma membrane-bound NADPH oxidases that catalyze the production of superoxide radical ($O_2^{\cdot-}$) from oxygen. The superoxide radical is a toxic reactive oxygen species (ROS) that enhance oxidative stress either by itself or by involving in the production of other ROS species such as the hydroxyl radical (OH^-) and hydrogen peroxide (H_2O_2). Zinc is important in production of metallothionein that is rich in cystine amino acid and considered to be an excellent scavenger of OH^-. Also, inflammatory cytokines, such as tumor necrosis factor (TNF) and interlukin-1B, produced by activated macrophages and known to generate ROS. Such inflammatory cytokines are found to be high in patients with low zinc concentration (Prasad, et al., 1993; Ozaki, et al., 1987; Prasad, et al., 2004).

1.4 Interactions of protein, zinc and iron during senescence, source-sink relations, phloem transport and seed deposition of nutrients

Senescence is the last stage of leaf development and induces remobilization of nutrients (simple sugars, amino acids and mineral nutrients) to the grain (Feller, et al., 1994; Marschner, 1995). Mobilization of photo-assimilates from mature leaves to the grain through natural senescence is a significant physiological process occurring at the generative stage of cereal crops. In general, micronutrient deficiency symptoms initiate in the young leaves and this phenomenon is explained by the absence of senescence at the early growth stages which could favor the transport of micronutrients from old to young plant parts (Marschner, 1995). The organic N content of wheat leaf is mainly composed of rubisco protein, which is almost totally hydrolyzed during leaf senescence. Prior to remobilization from leaves towards the grain, leaf proteins are firstly hydrolyzed to peptides and amino acids (Gepstein, 2004).

Simple sugars remobilized from shoot into grain during the senescence process are stored as starch in the endosperm whereas remobilized amino acids are used in synthesis of grain proteins (albumin, globulin, gliadin, glutenin) in the embryo, aleurone and the endosperm (Lasztity, 1996; Barneix, 2007). The most common limitation to protein synthesis is the low nitrogen availability. In general, an increase in the availability of nitrogen will lead to an increase in yield as well as the grain protein content. Grain proteins are synthesized at the end of the plant growth cycle; therefore; grain protein content is highly affected by the N supply rate (Spurway, 2008).

Since there is no grain-xylem connection in wheat all mineral and organic nutrients are transported via the floem (Welch, 1986; Pearson, et al., 1995). Also the high pH (7.5-8.0) of phloem is proposed to inhibit transport of cationic micronutrients and their transport in phloem is facilitated by chelating with organic ligands (Marschner, 1995). However, there is no detailed study in the literature addressing the transport forms of micronutrients in the phloem, particularly for Zn and Fe. It was proposed that nicotianamine and S-containing amino acids such as cysteine and methionine and their protein residues have a high Zn binding affinity. For this reason these compounds could be the main Zn carrier ligands in the phloem. During senescence large quantities of protein is hydrolyzed in the leaves and stems of matured wheat plants. Although there is no experimental evidence, it is proposed that Zn-amino acid ligands may play an important role in deposition of Zn into the grain (Von Wiren, et al., 1999; Dudev, et al., 2003; Haydon, et al., 2007; Torrance, et al., 2008). Also, reduction of grain protein, Fe, and Zn concentrations are related with decrease in their translocation from the leaves (Uauy, et al., 2006). Fisher et al have demonstrated that the composition of amino acids in the wheat phloem and in the wheat grain is similar (Fisher, et al., 1986).

It has been confirmed that high positive correlations exists among the grain concentrations of protein, Fe and Zn. Although durum wheat grain is harder and more adaptive to hot and dry conditions than bread wheat and contain more Zn, Fe and protein, it is not as rich as its wild progenitor emmer wheat (*Triticum dicoccoides*). *T. dicoccoides* has higher concentrations of Fe, Zn and protein; therefore; it became a feasible genetic resource to improve mineral and protein content of the cultivated wheat. Durum wheat nutritional quality is suggested to be enhanced by breeding and full use of the genetic diversity of Zn and Fe concentrations in synthetic and wild parents (Peleg et al,, 2008; Cakmak, et al., 2010; Ferney et al., 2010).

Availability and solubility of Zn and Fe in the soil is negatively affected by high soil pH, low moisture, low amount of organic matter and high CaCO3; but positively affected by high N, Zn and Fe-containing fertilizers. Supply of adequate N seems to be a prerequisite for higher root uptake and mobilization of Zn and Fe by increasing the expression level of Fe and Zn transporter proteins such as the *ZIP* family transporter proteins located on the root cell membranes. Fe and Zn are transported into the shoot through xylem vessels either chelated with a low-molecular organic compounds or free ions. N has also a positive role in the root-to-shoot transport of Fe and Zn either by chelating with nitrogenous compounds in the xylem such as nicotianamine and phytosiderophores or by increasing the levels of proteins contributing to xylem loading. Methionine is the precursor of nicotianamine. Zn and Fe transporter proteins located in the root cell membranes were also identified in the plasma membranes of the wheat phloem which may indicate their involvement in the Zn and Fe transport into seeds. Although high phloem pH may interfere with the Zn and Fe transport but their possible chelation with nicotianamine and amino acids may facilitate the process (Cakmak, et al., 2010).

Bioavailability and solubility of grain's Zn and Fe for humans are adversely affected by phytate, another grain component, but positively affected by the grain contents of cysteine, methionine and histidine, a well proposed sink for Zn and Fe (Cakmak, et al., 2010).

Zhao et al have found a very high positive correlation between both Fe and Zn and protein content among the bread wheat lines. They have suggested a possible link between these two trace elements and grain protein. They have found that the positive correlation between Fe and protein is higher than that of Zn and protein (Zhao, et al., 2009). Embryo and aleurone, the protein rich parts of the grain seed, are also rich in Zn where as the endosperm, which has low concentration of protein, is also low in Zn (Marschner, 1995). Ozturk et al, by using a Zn-staining method, had demonstrated that Zn is accumulated more in the embryo and aleurone than the endosperm (Ozturk, et al., 2006). Distelfeld et al have shown that the grain protein content-B1 (*Gpc-B1*) locus from wild emmer wheats affects the concentrations of both Fe and Zn and the grain protein content. The function of this locus is to encode *NAC* transcription factor (*NAM-B1*) which increases remobilization of nutrients from leaves to the grains by accelerating senescence. It is hypothesized that *Gpc-B1* locus increase the remobilization of micronutrients and proteins from senescing tissues into the seeds. However, the grain Zn concentration can, also be increased by delayed senescence by extending the

grain filling period in the presence of high nitrogen supply (Kutman, et al., 2010). There are three *NAM* genes in the wheat genome and modern wheat lines carry a non functional *aNAM-B1* allele which causes delayed leaf senescence resulting in decreased levels of grain protein, Zn, and Fe under limited N supply (Distefeld, et al., 2007; Cakmak, 2008).

1.5 Structure of Wheat Seed

Wheat seed is composed mainly of three parts that have different functions; bran, endosperm and germ. Bran is the brownish hard outer part of the grain. It protects the grain against whether changes, mold, insects, and bacteria. It consists of many layers that represent the concentrated source of dietary fiber in the grain. The layer of cells between the bran and the endosperm is called aleurone.

Aleurone is composed of single layer of cells surrounding the endosperm of the cereal seeds. It is a concentrated source of minerals, vitamins, proteins and other nutrients. This tissue synthesizes and releases some hydrolytic enzymes in response to gibberellic acids (GA3) in which α- amylase is the most abundant of all. There are two isoforms of α- amylase in wheat seeds that are encoded by two different structural genes. α- amylase is secreted into the starchy endosperm of the germinating seed where breakdown of the starch into maltose and glucose is accomplished (Bernal-Lugo, et al., 1999).

The endosperm is the inner part of the seed. It provides readily-usable energy and nutrients to the growing seedling. Endosperm is the main storage part of carbohydrates in the seed. Carbohydrates represent about 50-75% of the endosperm whereas 8-18% is consisted of protein. White flour is mainly produced from the endosperm by separating the bran and germ through milling processes.

Wheat germ contains the embryo and represents 2-3% of the total seed dry weight. Fatty acids, B and E vitamins are found in the germ.

Figure 1-1 illustrates the structure of wheat grain. The protein fraction of durum wheat and common wheat have a very high concentration of two amino acids, glutamate and proline, but very low for many essential amino acids such as threonine and lysine, and also low in tryptophan, isoleucine and methionine (Acquistucci, et al., 1995).

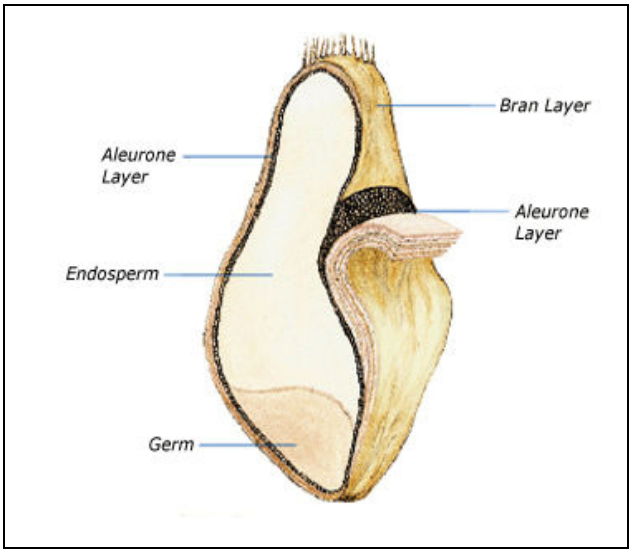

Figure 1-1 :Wheat grain parts (Milling, 2007)

The protein content of the wheat grain is distributed all over the kernel parts, but unevenly. The largest amount of protein is found in the endosperm, but the high concentration is found in the embryo. Table 1-1 shows the distribution and the concentrations of protein in the wheat grain parts.

Table 1-1: Wheat seed parts and the distribution of protein and starch as weight percent (Spurway, 2008)

Seed Part	% of seed weight	% of total starch	% of total protein
Bran (Pericarp)	8	0	4.5
Aleurone	7	0	15.5
Endosperm	82.5	100	72
Embryo (Germ)	2.5	0	8

1.6 Protein Hydrolysis

Proteins are composed of amino acids that are linked together via the peptide bonds. The amino group of a single amino acid molecule is attached with the carboxyl group of the second one. During peptide bonding the amino group loses a hydrogen atom and the carboxyl group loses both hydrogen and oxygen atoms yielding a molecule of water. It is for this reason the peptide bond is called a hydration bond (Ozman, et al., 2009).

The most accurate way of analyzing the total protein content is to precisely analyze all the individual amino acids and then take the sum to yield total protein content. However, the success of this process depends on the proper and complete hydrolysis of proteins into individual amino acids. Reproducible and quick separation of amino acids after breaking (or hydrolyzing) the peptide bonds helps in identifying, quantifying and characterizing the proteins (Weiss, et al., 1998). Amino acid analysis of a given material can not be expected to be successful without a proper hydrolysis step prior to analysis. There exist a number of protocols for protein hydrolysis that differ according to the end-use of the hydrolysates.

Usually, the hydrolysis is achieved by heating the sample in high concentrations of acids (usually HCl) using either thermal or microwave radiation energy. However, the success of this process depends on the proper and complete hydrolysis of proteins into amino acids.

The differences in the stability of amino acids are due to their side chains involvement in building the total structure as well as effect of the nonproteinaceous components which have a role in the hydrolysis conditions. The ultimate hydrolysis conditions are those that in one hand break all the peptide bonds and in the other hand cause no destruction of any amino acids (Zumwalt, et al., 1987).

1.6.1 Role of HCl concentration in amino acid recovery

Albin et al had investigated the effect of different HCl concentrations on the hydrolysis performance of Soya bean products. The recovery of certain amino acids was not affected with different HCl concentrations which include lysine, aspartic acid, threonine, and phenylalanine. Valine and isoleucine were recovered more by using HCl greater than 6 M, whereas histidine, glycine, arginine, alanine, leucine, proline, lysine, and phenylalanine were recovered more by HCl concentrations close to or lower than 6 M. Threonine recovery was maximized at 9 M HCl, however acid hydrolysis with 9M HCl resulted in degradation of tyrosine. Glutamic acid showed an increase in the recovery from 1 M to 3 M HCl but remain constant until 12 M HCl (Albin, et al., 2000).

Zhong et al had found that both microwave irradiation and acid type and concentration have an effect on peptide hydrolysis of membrane proteins. Short irradiation time (e.g., 2 min) and low acid concentration (e.g., 0.1 M HCl) resulted in fragments containing N-and/or C-terminus. Upon increasing irradiation time and acid concentration, more fragment ions as well as the N- and C- terminal fragment ions were found. At a longer irradiation time (e.g., 10 min) and higher HCl concentration (e.g., 1.5 M HCl) increased nonspecific cleavage formation was detected. Further increase in irradiation time and acid concentration generated hydrolytic peptides from both the internal fragmentation and the N- and C- terminus. Among strong acids, HCl does not react with the amino acids. Conversely, many other acids such as H_2SO_4 and HNO_3 react as oxidizing agents on amino acids whereas acetic acid modifies the N-terminus of peptides (Zhong, et al., 2005).

1.6.2 Protein oxidation before hydrolysis

Since acid hydrolysis can cause partial oxidation of amino acids, it is very important to optimize the hydrolysis conditions of hard samples such as cereal grains or feedstuff material,

particularly for the accurate quantification of sulfur-containing amino acids cystine and methionine. The oxidation of such materials prior to acid hydrolysis with a strong oxidant (i.e. performic acid) allows the accurate quantification of cystine as cysteic acid and methionine as methionine sulphone (Mason, et al., 1980). Although oxidation is very important in regard of accurate quantification of sulfur-containing amino acids, this inevitably results in oxidation of other amino acids (e.g. phenylalanine, tyrosine, histidine and arginine) and prevents their accurate quantification. For this reason, the practice of a sample oxidation step is adopted in many labs prior to classical HCl hydrolysis when sulfur-containing amino acids are needed to be analyzed using amino acid analyzer instruments.

1.6.3 Methods of protein hydrolysis

1.6.3.1 The classical reflux hydrolysis

The setup of a classical reflux hydrolysis is illustrated in
Figure 1-2. In this method, usually a small amount of sample (i.e. containing <10 mg N) is placed into a 100 ml bottom rounded Pyrex flask and added with 50 ml of 6 N HCl. The flask is then constantly heated at 110^{o}C for 24 hours during which the evaporated HCl is continuously condensed back (refluxed) in to the flask by cooling the flask neck. After 24 h of reflux, HCl is removed by lyophlisation, rotary evaporation or by drying down over sodium hydroxide. Then, if necessary, the sample is diluted with pH 2.2 loading buffer and filtered through 0.22 μm membrane filter (Messia, et al., 2008).

The classical method of protein hydrolysis by the reflux method is time consuming and low in productivity. However, it is currently the most widely accepted method by official organizations and legislations (EU Comisson directive 98/64/EC, 1998, AOAC Official Method 994.12, 1995) and has been extensively used to determine the level of hydrolyzed amino acid composition of samples from diverse origins (Basak, et al., 1993; Hirs, et al., 1954; Lupano, 1994, Lames and Fontaine, 1994).

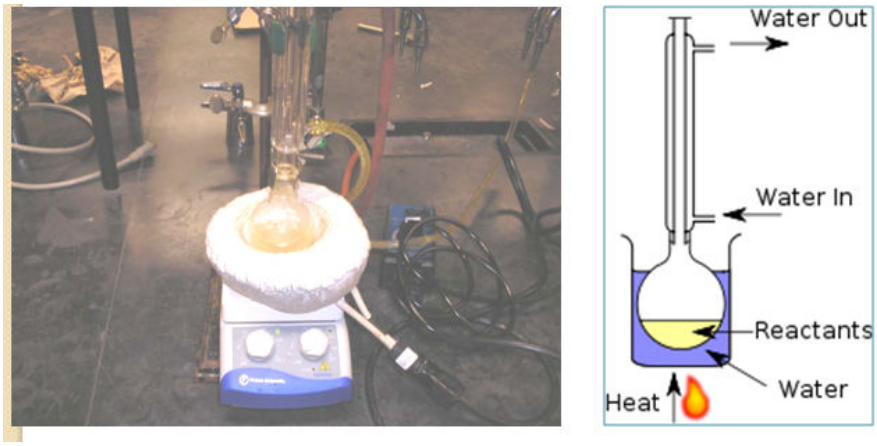

Figure 1-2: The setup of a classical reflux analysis.

1.6.3.2 Closed tube method

This method is similar to the open reflux method as far as the hydrolysis temperature and time, but the tubes containing the sample and the HCl are sealed under vacuum prior to hydrolysis. The sealed tubes are heated at 110^{o}C for 24 hours in temperature controlled ovens. The closed tube method is advantageous to open reflux for small amount of samples and also it is high in productivity. However, the recovery rates of the amino acids are very similar to the open reflux method (Pierce, 2006).

1.6.3.3 Microwave-assisted hydrolysis

Rapid hydrolysis of proteins coupled with high recovery rate of all of the amino acids offers a powerful tool in protein research, nutritional, and biochemical investigations. Roach et al had reported that protein hydrolysis of ribonuclease and bovine serum albumin for 4 hours at 145^{o}C had yielded comparable results with the classical reflux method (Roach, et al., 1970). Microwave technology offers cheap, clean, and convenient method for heating which results in high recovery and shorter times of reaction (Phani, et al., 2006).

Microwaves are electromagnetic radiation locating in the electromagnetic spectrum between the infrared and the radio waves with the following characteristics: wavelength (in centimeters): 10 - 0.01, frequency (in Hertz): 3 x 109 - 3 x 1012, and energy (in electron volt): 10-5 - 0.01 (Pozar, 1997). The microwave irradiation theory was predicted in 1864 but physically demonstrated in 1888. Magnetron, the high-energy machine that was used to generate microwave energy, was invented as part of the radar detection system during the World War II. In 1946, the microwave irradiation was discovered as a heating method. The first commercial microwave was introduced in 1950s. Over the past three decades, a lot of improvements have been done to the laboratory microwaves to include models specific for polymer synthesis, peptide synthesis and process control. Figure 1-3 illustrates an up-to-date laboratory microwave system that is capable of heating up to 40 closed vessels per run with self regulated pressure control and processor controlled temperature.

Figure 1-3: A modern laboratory microwave reaction system by CEM Co. Matthews, US (Model: MARSXpress).

The mechanism of microwave catalysis depends on three main categories: dipole rotation/polarization, conduction, and interfacial polarization which all cause agitation of

polar molecules and thus increase their temperature without causing rearrangement of molecular structure (Yao, et al., 2008).

In previous studies microwave radiation was used to conduct hydrolysis of purified proteins like bovine serum albumin or methionyl human growth hormone (m-HGH Protropin1) and exposing protein or acidic peptide solutions to microwave irradiation accelerated release of amino acids and thus decreased the time of the hydrolysis procedure (Pecavar, et al., 1990; Lill, et al., 2007). Precision and accuracy that were achieved in short-time microwave-assisted hydrolysis (5-15 min) were almost equivalent to those that were achieved by using conventional heating at 110^{o}Cfor 24 h (Lill, et al., 2007 and the references therein). Evidence from literature indicates that microwave-assisted hydrolysis of proteins yields comparable results to that of the classical open reflux hydrolysis. However, microwave hydrolysis is expected to replace the open reflux or sealed tube methods due to significant reduction in hydrolysis time, energy and chemical reagent consumption.

To our knowledge, microwave-assisted hydrolysis was not studied in complex samples such as cereal grains, flour or feedstuff that are rich in carbohydrates and dietary fiber. Therefore, part of this thesis study includes optimization of a new expedient method for microwave-assisted hydrolysis of wheat proteins and amino acid profiling of wild, primitive and cultivated wheat. Correlations between the resulting amino acid profiles and Zn, Fe and protein concentrations in different wheat species were analyzed and discussed in a separate chapter.

1.6.3.4 Enzymatic digestion

Peptide bonds are cleaved by proteolytic enzymes that have specific and well-defined activities as carboxypeptidase, trypsin, chymotrypsin, thermolysin and papin.
Enzymatic hydrolysis has the advantage of total and full amino acid recovery including asparagine and cystiene (which are usually destroyed in both conventional heating method and microwave-assisted method), also it is preferred when sequencing of certain proteins is required. This method is not widely applied, especially for the unpurified proteins, due to hard accessibility. Usually, it takes a long time, 18-24 hours, and requires many enzymes to accomplish a full hydrolysis. Enzymes are usually expensive. (Fountoulakis, et al., 1998).

1.6.3.5 Alkaline hydrolysis

During acid hydrolysis tryptophan is totally degraded by HCl, and the commonly used way to recover it is by the alkaline hydrolysis method, which is also used when the sample contain high amount of carbohydrates. Alkaline hydrolysis is usually performed with either NaOH or KOH, and, rarely, with barium hydroxide. The major disadvantage of this method is the destruction of threonine, serine, cysteine and arginine. Therefore, alkaline hydrolysis methods are almost dedicated to recover tryptophan only (Fountoulakis and Lahm, 1998).

1.7 Amino Acids

Amino acids are important units of all organisms from bacteria to mammals. They are bound together to form proteins which are vital to life. An optimum protein intake provides all the 20 amino acids, essential for both human and animal life, in the correct proportions to fulfill the diverse needs of the body for metabolic functions including modulation of gene expression (Palis, et al., 2009), intestinal integrity (Wang, et al., 2009) and protein synthesis (Suryawan, et al., 2009).

Amino acids are structures that contain amine group, carboxylic acid and side chain. The key elements are oxygen, hydrogen, nitrogen and carbon. There are many different amino acids, but the important ones to living processes are only 20, and around 10 of them are essential to human body. High number of amino acids is bound together by peptide bonds to form large polypeptides (proteins). The analysis of the amino acids can be realized following liberation of the peptide bonds by hydrolysis (Johnson, et al., 1958).

1.7.1 Features of amino acids

The amino acids are crystalline solids with high melting points. Their melting and decomposition tend to be in the range of 200-300^{o}C (Clark, 2007). Amino acids are organic compounds that have both carboxylic acid -COOH and amine group –NH2 (therefore; they

are called amino acids). Both groups are attached to a carbon atom, by which hydrogen atom and side chain attached. The side chain (called R group) gives each amino acid its unique properties. Also, this side chain gives each amino acid its specific charge distribution which is used as the basis for the ion exchange chromatography. Amino acids are different in their recovery upon protein hydrolysis at any given temperature. Proline, threonine, methionine, arginine and serine are the most sensitive to heat. Leucine, isoleucine and valine are the most stable amino acids and require about 70 h at 110^{o}C for maximum recovery (Roach, et al., 1970). So far, no method is introduced that is capable of fulfilling both complete recovery and zero degradation of all individual amino acids.

Proline and hydroxyproline, the imino acids, have no primary amino groups. Nitrogen reacts with the R group forming a five-membered pyrrolidine figure. Most amino acids at pH 7 are dipolar ions (zwitterions), the carboxyl group loses its hydrogen and the amino group is protonated. Another feature about amino acids is that all of them are chiral except glycine. They superimpose their mirror image and they exist in either mirror image. One mirror image is termed D (dextro or right) and the other mirror image is termed L (laevo or left) (Rawn, 1989; Johnson, et al., 1958). The names, symbols, and structures of amino acids and structure of the side chains are shown in Table 1-2 and Table 1-3.

Table 1-2: Classification of the common amino acids based on the chemistry of the R group (Condon, 1986)

ALIPHATIC AMINO ACIDS	NON-ALIPHATIC AMINO ACIDS
Monoamino-dicarboxylic acids Glutamic acid Aspartic acid	**Aromatic Amino Acids** Tyrosine Phenylalanine
Hydroxy-monoamino-monocarboxylic acids Serine Theronine	**Monoamino-dicarboxyl-co-amides** Aspargine Glutamine
Monoamino-monocarboxylic acids Alanine Glycine Isoleucine Valine Leucine	**Diamino-monocarboxylic acids** Lysine Arginine Ornithine
	Heterocyclic Amino Acids Histidine Tryptophan Proline, Hydroxyproline Tryptophan
	Sulphur-Containing Amino Acids Cystine Methionine

Table 1-3: Amino acid structures (Condon, 1986)

Amino Acid	Symbol	Structure	Formula Weight
		Aliphatic Amino acids	
Glycine	Gly(G)	NH_2-CH_2-COOH	75.07
L-valine	Val(V)	$(CH_3)_2$-CH-CH(NH_2)-COOH	117.15
L-alanine	Ala(A)	CH_3-CH(NH_2)-COOH	89.09
L-leucine	Leu(L)	$(CH_3)_2$-CH-CH2-CH(NH_2)-COOH	131.17
L-isoleucine	Ilu(I)	CH_3-CH_3-CH(CH_3)-CH(NH_2)-COOH	131.17
L-aspargine	Asn(N)	H_2N-CO-CH_2-CH(NH_2)-COOH	132.12
L-proline	Pro(P)	NH-$(CH_2)_3$-CH-COOH	115.13
L-glutamine	Gln(Q)	H_2N-CO-$(CH2)_2$-CH(NH_2)-COOH	146.15
		Sulphur-Containing Amino Acids	
L-methionine	Met(m)	CH_3-S-$(CH2)_2$-CH(NH_2)-COOH	149.21
L-cysteine	Cys c	HS-CH_2-CH(NH_2-COOH-COOH	121.16
		Hydroxylated-Amino Acids	
L-threonine	Thr(t)	CH_3-CH(OH)CH(NH_2)-COOH	119.12
L-serine	Ser(s)	HO-CH_2-CH(NH_2)-COOH	105.09
		Aromatic Amino Acids	
L-phenylalanine	Phe(p)	C_6H_5-CH_3-CH(NH_2)-COOH	165.19
L-tryptophan	Trp(w)	C_6H_4-NH-CH=C-CH_2-CH(NH_2-COOH	204.23
L-tyrosine	Tyr(y)	HO-C_6H_4-CH_2-CH(NH_2)-COOH	181.19
		Acidic Side Chains	
L-glutamate	Glu(e)	HOOC$(CH_2)_2$-CH(NH_2)-COOH	147.13
L-aspartate	Asp(d)	HOOC-CH_2-CH(NH_2)-COOH	133.1
		Basic Amino Acids	
L-lysine	Lys(k)	CH_3-CH_3-CH(CH_3)CH-NH_2-COOH	146.19
L-arginine	Arg r	HN=C(NH_2)-NH-$(CH_2)_3$CH(NH_2)-COOH	174.2
L-histidine	His (h)	NH-CH=N-CH=C-CH_3-CH(NH_2)-COOH	155.16

Amino acids biosynthesis is controlled by the nucleotide sequence of the DNA and the corresponding mRNA. The individual codons and their corresponding amino acids are listed in Table 1-4.

Table 1-4: Amino acid genetic code (Ozman, et al., 2009)

TTT Phe	TCT Ser	TAT Tyr	TGT Cys
TTC Phe	TCC Ser	TAC Tyr	TGC Cys
TTA Leu	TCA Ser	TAA Stop	TGA Stop
TTG Leu	TCG Ser	TAG Stop	TGG Trp
CTT leu	CCT Pro	CAT His	CGT Arg
CTC Leu	CCC Pro	CAC His	CGC Arg
CTA leu	CCA Pro	CAA Gln	CGA Arg
CTG Leu	CCG Pro	CAG Gln	CGG Arg
ATT Ile	ACT Thr	AAT Asn	AGT Ser
ATC Ilu	ACC Thr	AAC Asn	AGC Ser
ATA Val	ACA Thr	AAA Lys	AGA Arg
ATG Met	ACG Thr	AAG Lys	AGG Arg
GTT Val	GCT Ala	GAT Asp	GGT Gly
GTC Val	GCC Ala	GAC Asp	GGC Gly
CTA Val	GCA Ala	GAA Glu	GGA Gly
GTG Val	GCG Ala	GAG Glu	GGG Gly

1.7.2 Zinc-binding amino acids

Almost one-third of the proteins that are defined in the Protein Data Bank (PDB) contains metals and therefore named as metalloproteins. Metals play a critical role in the functions, structure and stability of metalloproteins (Bernstein, et al., 1977). In eukaryotic organisms, Fe and Zn are the most abundant metals playing catalytic and structural roles in many biological functions (Coleman, 1992).

Shu et al have developed a method to predict the zinc-binding sites in proteins by combining homology-based predictions and support vector machine (SVM). Their method has predicted zinc-binding Histidine, Cysteine, Glutamic acid, and Aspartic acid with 75% precision (Shu, et al., 2008).

1.7.3 Amino acid analysis techniques

1.7.3.1 Historical perspective

The major breakthrough in the field of chemistry and biochemistry of amino acids, peptides, and proteins was achieved in 1910 by Siegfried Ruhemann by revealing the ninhydrin reaction. He wrote, "The further study of triketohydrindene hydrate led to results which appear to be of great interest. It was found that a deep blue color is produced on warming a mixture of aqueous solutions of this compound with aliphatic or an aliphatic-aromatic amine-which contains the amino group in the side chains" (Ruhemann, 1910). One year later in 1911 Abderhalden and Schmidt had collaborated with Ruhemann and studied the reaction of a large number of different compounds with this reagent in order to determine the extent to which the reaction is typical with different classes of compounds (Abderhalden and Schmidt, 1911). Among 26 compounds that had been investigated, 2 proteins and 23 amino acids produced typical blue-purple color, but the color was yellow with proline. These initial observations were followed by further studies to extend the usefulness of the ninhydrin reaction (Abedrhalden and Schmidt, 1913; Ruhemann, 1910). In the following years studies with ninhydrin influenced many scientists to explore its reaction with amino acids. The most important advancement in the history of the ninhydrin reaction was probably the automation of chromatography in 1958 by Stein and Moore (Stein and Moore, 1958). This had helped in enabling quick assays of all amino acids in protein hydrolysates at nanomole levels (Moore, 1968).

An important contribution to ninhydrin detection came from Dent. It was the use of ninhydrin sprays to develop 60 ninhydrin-positive compounds on thin layer paper chromatograms which, nowadays, are widely used with paper and silica gel plates (Condon, 1986).

Stein and Moore had spent a considerable amount of time in the separation of amino acids. In 1958, along with Spackman, they published their view of an automated instrument for the separation and quantitative of compounds that were ninhydrin-positive (Spackman, et al., 1958).In 1972, they were awarded the Noble Prize for their contribution to the understanding of the connection between chemical structure and catalytic activity of the active centre of the ribonuclease molecule.

Since 1958, many authors had published improvements, but not basic changes to this technique. These papers showed the effort and success toward faster and more sensitive analyses (Piez, et al., 1960; Bohlen, et al., 1982).Also, there have been improvements to the detection reagent, 2-methoxyethanol that was used until Moore replaced it by dimethylsulphoxide (Moore, 1968). Since 1981, ethylene glycol was recommended by Biochrom Co., UK to become the solvent for ninhydrin, and nowadays it is widely used.

Fluorescent reaction with amino acids was first investigated with a fluorophore called fluorescamine. This compound was not widely used because of the development of an improved and cheaper fluorophore, o-phthaladehyde (OPA). Since both reagents (OPA and fluorescamine) do not react with imino acids, sodium hypochlorite was used to oxidize imino acids before reaction with OPA to end up with a fluorescent product. Since hypochlorite partially oxidizes some of the amino acids, it is introduced during the elution of imino acids (Condon, 1986; Davis, 1986).

1.7.3.2 Paper chromatography

As all kinds of chromatography, in paper chromatography there is a stationary phase and a mobile phase. Amino acids can be separated on layers of cellulose paper. This is an economical method as well as it has been widely used. Several samples can be run at the same time and then visualized by spraying ninhydrin, diaminobenzaldehyde or trinitrobenzensulphonic acid. This method is qualitative but not quantitative (Chemguide, 2007; Davis, 1986).

1.7.3.3 Thin-layer chromatography

The thin-layer chromatography (TLC) method depends on the partition of a solute (e.g. protein hydrolysate) between a moving and a stationary phase. Usually the moving phase is an organic solvent, and the stationary phase is a layer of water attached to a solid support-silica gel or aluminum oxide. The first step is loading a small spot of protein hydrolysate on the stationary phase. The second step would be inserting the plate in a moving phase which is a chamber containing a layer of organic solvent. The protein hydrolysate sits just above the organic solvent. As the organic solvent moves across the plate by capillary action and touches the stationary phase, the amino acids will be separated between the stationary phase and the organic solvent. Those amino acids that have higher affinity to silica gel will stay in the stationary phase or move slowly up the plate, where as the others that have higher affinity to the organic solvent (e.g. hydrophobic amino acids) will move quickly up the plate (Rawn, 1989).

1.7.3.4 Electrophoresis

Separation of amino acids based on their charge is possible by electrophoresis. The solution has certain pH at which some amino acids will migrate toward the negative electrode or toward the positive one and the amino acids that are neutral at that pH will not move. As an example, at pH 7, alanine is neutral and will not move, arginine is positive, and glutamic acid is negative. Consequently, the number of amino acids that are investigated by electrophoresis is limited (Davis, 1986).

1.7.3.5 Ion-exchange chromatography

This method depends on the differences in net charges of the amino acids at a certain pH. In ion-exchange chromatography, the stationary phase consists of an insoluble matrix (e.g. synthetic resin granules) packed in a column. The resin is named either a cation-exchange resin or an anion-exchange resin according to the charged groups attached to the matrix granules. The pH of the buffer that is used to elute the column may have a role in the charge of the resin. Once the protein hydrolysate is added to a column filled with cation-exchange

resin, ions that have greater positive charge bind stronger to the resin than those of the negative ones. Since amino acids have different charges at a given pH, the amino acids elute at different times (Rawn, 1989).

1.7.3.6 High-performance liquid chromatography (HPLC)

High-performance liquid chromatography (HPLC) has replaced ion-exchange and thin-layer chromatography in many laboratories. In HPLC the eluent flow is pushed under a hydrostatic pressure of 350-700 kg cm^{-2} through a stainless steel column. The driving force of hydrostatic pressure is achieved by high pressure pumps which increase the performance of regular column chromatography that uses gravity as the driving force. In HPLC column, the particles are very finely divided with excellent mechanical strength. The separations obtained in HPLC are much better than in the conventional chromatography and smaller samples can be analyzed (Rawn, 1989).

1.7.4 Biochrom-30 amino acid analyzer

The Biochrom-30 amino acid analyzer is actually an HPLC instrument dedicated for quantification of amino acids. The sample contain a mixture of amino acids is loaded to a column filled with cation-exchange resin. The amino acids are separated by buffers of different pH and ionic strength (mobile phase) that are pumped into the column (stationary phase). The column temperature is controlled accurately to produce the required separation.

The resin in the column has a negative charge and the amino acids are loaded to the resin mixed in low pH citrate buffers (i.e. pH 2.2-4.25) ensuring all amino acids to be positively charged for a strong binding to the resin. The use of buffers with different pH enhances separation of amino acids by changing the isoionic points of individual amino acids. A picture for the Biochrom 30 amino acid analyzer is illustrated in
Figure 1-4.

As an example, the ionization state of aspartic acid as a function of pH is shown in
Figure 1-1 (Condon, 1986). At pH 1 aspartic acid has a net positive charge and as the pH increases to 2.8 the α carboxyl group loses a proton, thus the molecule gets negatively charged and called to be at the isoionic state. The carboxyl group in the side chain is less

acidic than α carboxyl group and the hydrogen ion concentration is still enough to prevent its ionization. When the pH reaches 6.6 the side chain carboxyl group is now ionized with two negative charges and one positive charge. At pH 11 the molecule has two negative charges and no positive one (Condon, 1986).

Figure 1-4: Biochrom 30 amino acid analyzer

Another example is the lysine in which the side chain is an amino group. The isoionic point is 9.7 and it is the side chain amino group that gets charged at pH 9.7, it is more basic than the α amino group as illustrated in Figure 1-6.

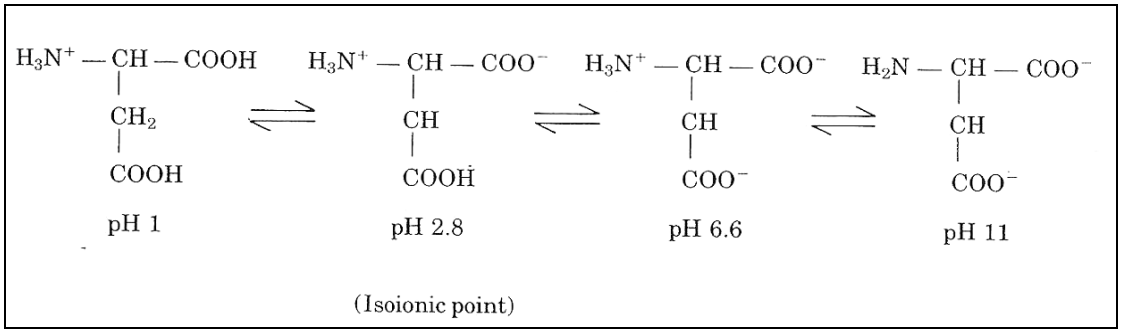

Figure 1-5: Ionization state of aspartic acid as a function of pH (Condon, 1986)

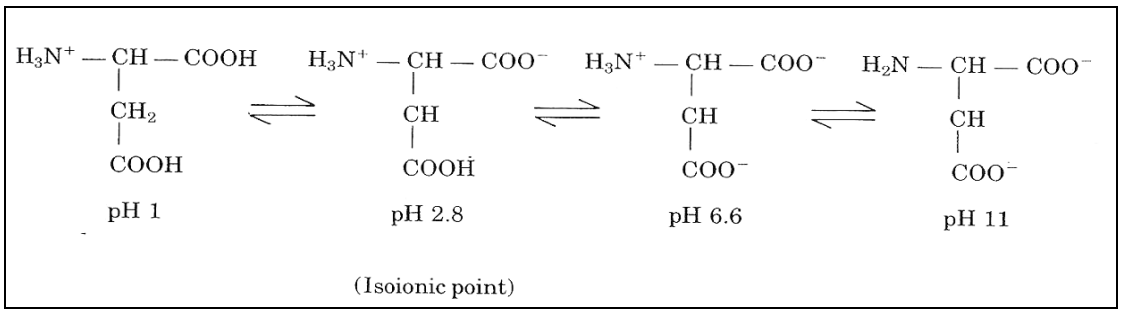

Figure 1-6: Ionization of lysine as function of pH (Condon, 1986)

The temperature of the analytical column plays an important role in the resin amino acid reaction and therefore precisely controlled with peltier elements. Increasing the column temperature decreases the retention time (the time that amino acids stay in the column) for the amino acids. Also, the addition of the organic solvents to buffers helps in separation of some amino acids. They change the affinity of the amino acids to the resins, which then changes the retention time. For example, both threonine and serine have similar isoionic points but their side chains are different, threonine has one extra methyl group which makes it more hydrophobic. The resolution between those amino acids can be improved by addition of 1-2% isopropanol to the first analytical buffer which causes faster elution for the more hydrophobic amino acids (Condon, 1986).

The ion exchange process that occurs in the Biochrom amino acid analyzer can be summarized as:

$SO_3\ Na^+$—MATRIX—H_3^+N—CHR—COOH= MATRIX—$SO_3^-H_3^+$N—CHR—$COOH^+\ Na^+$

Separation of the amino acids from the matrix is influenced by increasing the pH to move the equilibrium towards the left.

The resin used in Biochrom amino acid analyzer is composed of spherical polystyrene beads. The chains of the polystyrene beads are cross linked by divinylbenzene groups (DVB) and are also sulphonated to provide them with negative charge.

The ninhydrin is pumped to the heated reaction coil to get mixed with eluent. In the reaction coil, the amino acids react with the ninhydrin to form colored compounds which are directly proportional to the quantity of the amino acid present. The eluent-ninhydrin mixture present in the reaction coil is then pumped to the photometer unit to determine the amount of each colored compound by measuring the amount of light absorbed at two wavelengths, 440 nm and 570 nm.

The photometer output is linked to 2 channels computer-based integration system. One channel for the output of the 440 nm photometer, and the other is for the output of the 570 nm photometer. The concentrations of the amino acids are recorded as a series of peaks. Each amino acid is identified by its retention time, and the quantity of each amino acid is indicated by the area under the peak. Since the amino acid analyzer is a comparative instrument, a calibration run should be performed with known standards at regular intervals (e.g. in every batch of 15-20 analysis). Following each sample analysis, the column is flushed by pumping a strong base through it followed by a low pH buffer, which equilibrates the column before the next analysis (Davis, 1986).

1.7.5 Detection systems in amino acid analyzers

1.7.5.1 Fluorescence detection

Fluorescence detection is a highly sensitive method where the fluorescence intensity is proportional to the quantity of the light absorbed and the photoluminescence efficiency. Only few of the common amino acids have their own fluorescence, and in order to form a fluorescent compound, amino acids are required to be reacted with fluorogenic reagent. The fluorogenic reagents that are widely used in amino acid analysis are fluorescamine and OPA. Usually, the detection of the fluorophores takes place with an excitation filter between 340 nm and 370 nm and a long pass emission filter of 425 nm.

Fluorescence detection systems have many disadvantages which need to be overcome for maximum response. Such disadvantages as:

- System overheating (insufficient cooling of the source).
- Isoindoles structures are formed in the fluorogenic reaction of OPA and amino acids. They have excitation maximum at 280 nm (below the range of some sources).
- Fixed slit widths do not allow optimization of sensitivity and reduction in stray light.

1.7.5.2 Ninhydrin detection

Ninhydrin is a strong oxidizing agent and leads to the oxidative deamination of the amino group freeing ammonia, carbon dioxide, aldehyde with one less carbon atom, and hydrindantin (reduced form of nihydrin). Ammonia, one of the outputs of the ninhydrin reaction with amino acids, reacts with hydrindantin and another molecule of ninhydrin to produce a purple substance called Ruhemann's purple with an absorption maxima at around 570 nm. This absorbance is a linear function of the amount of the α-amino groups present enabling quantitative and convenient colorimetric assay for all organic compounds with the α-amino groups. The reaction is catalyzed at high temperatures, and Biochrom analyzer has post column reactor that is controlled at $135C^{o}$ for short time. This allows stable and reproducible detection. It looks like that hydrindantin is required in the detection reagent to stop a side reaction that may reduce the amount of Ruheman's purple formed (Lamothe, et al., 1973).

Upon reaction of ninhydrin with the imino acids (that do not have free amino groups), such as proline and hydroxyproline, the product will be bright yellow with an absorption maxima at around 440 nm. Measurement of the imino groups below 440 nm does not provide true readings with a false response due to the absorbance of unreacted ninhydrin (Davis, 1986).

1.7.6 Interpretation of amino acid analysis

Amino acid analysis plays a major role in protein chemistry as it supplies the initial and important information for the analysis of the protein structures. By using recombinant DNA

technology many different proteins can be produced. At this point, amino acid analysis is significant to confirm the predicted structure. Post-transitional modifications generate hundreds of different amino acids. Such modifications include phosphorylation, acetylation, nitration, hydroxylation and others. Those amino acids are distinguished from the naturally occurring amino acids by retention time and also by 570/440 peak area ratios e.g. iodination and hydroxylation reduce the 570/440 ratio, and phosphorylation reduces the retention time (Hodisan, et al., 1998; Kadowaki, 1993).

In humans, analysis of amino acid profiles allows assessment of nutritional and health disorders. It detects the classic homozygote amino acid disorder and gives an indication about the secondary disorders as well as mildly expressed inborn errors (Briddon, et al., 1987). Also, it helps in diagnosis and treatment of nervous and mental diseases caused by problems in metabolism of amino acids such as increased hydroxyproline, hyperlysinemia and hyperglycinemia may indicate mental retardation, high valboric acid, leucine, and isoleucine may cause coma (Lakhan, et al., 2008; Bazinet, et al., 2006).

1.7.7 Ninhydrin pharmacology and toxicology

Upon ingestion by laboratory animals and cell cultures, ninhydrin has induced adverse and beneficial effects. It appeared to promote carcinogenesis in the multistage mouse skin carcinogenesis assay. Tumor promotion was accompanied by high activity of the enzyme γ-glutamyl transpeptidase. But it appeared to be as anti-carcinogenic in Ehrlich ascitis carcinoma cells. Ninhydrin gave protection against the damage of the mucosa in the rats that may be induced by ethanol. The ninhydrin anti-ulcergenic activity may be due to its characterstic of oxidizing the SH groups to disulphide bonds. (Friedman, 2004; Al-Shabanah, et al., 2000).

Although ninhydrin has not been proved to be diabetogenic, at low concentration it was toxic to rat beta cells, and at high concentrations destroyed all the islet cells. Ninhydrin inhibited the enzyme glucokinase in the pancreatic cells, probably by oxidation of SH groups of the enzyme. Ninhydrin destroys the pancreatic cells before it causes diabetes (Friedman, 2004)
It has been reported that ninhydrin decreased the activity of the antioxidative enzymes (glutathione peroxidase, catalase, superoxide dismutase) in pancreatic cells, inhibited the

aconitase enzyme in the liver cells, and caused neurological changes in rodents (Picton, et al., 2002).

The observations cited by many scientists suggest that laboratory workers and others should take precautions and avoid exposure to ninhydrin (Friedman, 2004).

1.7.8 Utilization of wheat proteins as food and feed

Wheat grains contain little amount of protein with an average of about 6-16 % dry weight Nevertheless; they provide over 60 million tons of proteins annually for the nutrition of livestock and humans (Uauy, et al., 2006). Beside its nutritional importance, wheat seed proteins also promote the utilization of the grain in food processing; it is consumed by human after processing into bread and other foods. It is not surprising, therefore, that wheat proteins have been a hot topic of research for over two hundred years (Shewry, et al., 2002).

As mentioned earlier, wheat proteins are composed of four different types of proteins; albumins, globulins, gliadins and glutenins. Glutamine and proline amino acids, constitute about 30-70% of the whole wheat proteins, form a viscoelastic network in dough and are responsible for the ability to process wheat to form food product such as bread and pasta (Shewry, et al., 2002).

Normal growth of humans and maintenance of their health require all amino acids to be provided in suitable quantities as well as in biologically utilizable forms (bioavailability). All of the amino acids are supplied by wheat with variable concentrations. The most limiting amino acid in wheat is lysine (Elango, et al., 2009).

Proteins are hydrolyzed in the stomach by proteases such as trypsin, chymotrypsin, pepsin, pepsinogen and others. Upon hydrolysis, amino acids are utilized in the body to help in different aspects. For example, they are utilized in hormones that influence the metabolism, utilized to make enzymes that catalyze biochemical reactions. Also, they are utilized to make hemoglobin to carry oxygen throughout the body and antibodies that help in fighting the infections and building the immune system. Amino acids even have role in building and repairing ligaments, muscles, tendons, organs, glands and nails. Moreover, some of them act as neurotransmitters to carry messages within the neurons. In other words, all of the amino

acids are important to human body functions and a deficiency in just one of them can negatively and severely affect our health (Spurway, 2008; Kimball, 2009; Rhoads, et al., 2009).

2 Materials and Methods

2.1 Reagents, solutions and buffers

Sodium citrate buffers (pH 2.2, 2.65, 3.35, 4.25 and 8.6) and ninhydrin solution were purchased from Biochrom Co., UK. Ninhydrin solution was prepared by mixing Ultra ninhydrin and Ultra Solve plus solutions and purged by N_2 for 10 minutes as advised by the producer company Biochrom, UK. Amino acid standard was prepared by diluting the Sigma AAS18 standard with sodium citrate loading buffer (pH 2.2) to yield 5 nmol 20 μL^{-1} for each amino acid. 6 N HCl solution was prepared by adding 497 ml concentrated HCl (37%) to 503 ml deionized water. Performic acid was prepared by adding 900 ml 88% formic acid with 100 ml 30 % H_2O_2. All chemicals and buffers that were used are listed in Appendix A.

2.2 Equipments

All equipments that were used are listed in Appendix B.

2.3 Materials

Seeds of modern bread and durum wheats (*T. aestivum* and *T. durum*), primitive spelt wheats *(Triticum aestivum ssp. spelta*) and wild emmer wheats (*T. dicoccoides*) were kindly provided by Cukurova University Research Farm and Prof. İsmail Çakmak. The spelt wheats were selected for either high or low grain N content (i.e. four low-N and five high-N genotypes) whereas modern and wild emmer wheats were selected randomly. Following drying at 40^{o}C for 2 h all seeds were milled in a vibrating agate cup mill (Pulverisette 9, Fritsch GmbH, Idar-Oberstein, Germany) for 5 min at 700 RPM. The resulting whole meal wheat flour was used in the experiments. In the optimization of the non-oxidized amino acid experiments *T. durum* cv. Balcali 2000 was used, whereas in the optimization of the oxidized amino acid experiments a standard reference material (SRM 8436 Durum Wheat Flour, National Institute of Standards and Technology, Gaithersburg, USA) was used. In experiments conducted to

reveal the relationships between amino acids, N, Fe and Zn, all seed materials were evaluated. A list of the seed materials used in all experiments is given in Table 2-1.

Table 2-1:List of seed material used in the experiments

Genotype	Species/subspecies	Used in experiment
Balcali 2000	*T. durum*	AA profiling and correlation with nutrients, optimization of non-oxidized AA
Tuten	*T. durum*	AA profiling and correlation with nutrients
Gediz	*T. durum*	AA profiling and correlation with nutrients
EGE 2005	*T. durum*	AA profiling and correlation with nutrients
Zenit	*T. durum*	AA profiling and correlation with nutrients
Meram	*T. durum*	AA profiling and correlation with nutrients
Yelken	*T. durum*	AA profiling and correlation with nutrients
Kumbet	*T. durum*	AA profiling and correlation with nutrients
Selcuklu	*T. durum*	AA profiling and correlation with nutrients
Yilmaz	*T. durum*	AA profiling and correlation with nutrients
Karahan	*T. aestivum*	AA profiling and correlation with nutrients
Adana 99	*T. aestivum*	AA profiling and correlation with nutrients
Ahmetaga	*T. aestivum*	AA profiling and correlation with nutrients
Alpu 1	*T. aestivum*	AA profiling and correlation with nutrients
Gerek	*T. aestivum*	AA profiling and correlation with nutrients
Bezostaja	*T. aestivum*	AA profiling and correlation with nutrients
Tosun bey	*T. aestivum*	AA profiling and correlation with nutrients
Kirgiz	*T. aestivum*	AA profiling and correlation with nutrients
C-1252	*T. aestivum*	AA profiling and correlation with nutrients
Sp 211	*T. aestivum ssp. spelta*	AA profiling and correlation with nutrients
Sp 207	*T. aestivum ssp. spelta*	AA profiling and correlation with nutrients
Sp 89	*T. aestivum ssp. spelta*	AA profiling and correlation with nutrients
Sp 21	*T. aestivum ssp. spelta*	AA profiling and correlation with nutrients
Sp 663	*T. aestivum ssp. spelta*	AA profiling and correlation with nutrients
Sp 244	*T. aestivum ssp. spelta*	AA profiling and correlation with nutrients
Sp 926	*T. aestivum ssp. spelta*	AA profiling and correlation with nutrients
Sp 818	*T. aestivum ssp. spelta*	AA profiling and correlation with nutrients
Sp 804	*T. aestivum ssp. spelta*	AA profiling and correlation with nutrients
TD 536	*T. dicoccoides*	AA profiling and correlation with nutrients
TD 531	*T. dicoccoides*	AA profiling and correlation with nutrients
TD 510	*T. dicoccoides*	AA profiling and correlation with nutrients
TTD 27	*T. dicoccoides*	AA profiling and correlation with nutrients
TD 195	*T. dicoccoides*	AA profiling and correlation with nutrients
TD 391	*T. dicoccoides*	AA profiling and correlation with nutrients
TTD 28	*T. dicoccoides*	AA profiling and correlation with nutrients
TD 390	*T. dicoccoides*	AA profiling and correlation with nutrients
TTD 89	*T. dicoccoides*	AA profiling and correlation with nutrients
TD 636	*T. dicoccoides*	AA profiling and correlation with nutrients
TTD 86	*T. dicoccoides*	AA profiling and correlation with nutrients
TTD 75	*T. dicoccoides*	AA profiling and correlation with nutrients
TD 399	*T. dicoccoides*	AA profiling and correlation with nutrients
TTD 18	*T. dicoccoides*	AA profiling and correlation with nutrients

2.4 Methods

2.4.1 Mineral nutrient analysis of wheat flour samples

Whole meal flour of wheat genotypes were analyzed for K, P, Mg, S, Ca, Fe, Mn, Zn and Cu concentrations by ICP-OES (Vista-Pro Axial; Varian Pty Ltd, Mulgrave, Australia) following acid digestion by a microwave digestion system (MarsExpress; CEM Corp., Matthews, NC, USA). Flour N concentration was measured by an automated N analyzer (TruSpec CN, LECO Corp., Michigan, USA). Protein concentration was calculated by multiplying the N concentration by 5.83 (Merrill and Watt, 1973) as needed. All results were validated by the certified values of a standard reference material (SRM 8436 Durum Wheat Flour, National Institute of Standards and Technology, Gaithersburg, USA). The same standard reference material (SRM 8436 Durum Wheat Flour) was also used in a round robin amino acid analysis in which two independent laboratories (i.e. Biochrom Co. and Ansynth Service B.V. laboratories) participated by utilizing the traditional 24 h reflux hydrolysis with 6 N HCl to compare the results of the oxidized and non-oxidized hydrolysis experiments. Currently, the 24h reflux method is known as the most common method of protein hydrolysis that yields high recovery of amino acids (Basak, et al., 1993; Lupano, 1994). In view of its reproducibility and good yields, the method has been adopted by the EU and by many other countries as the accredited method of protein hydrolysis. The two laboratories Biochrom Co. and Ansynth Service B.V. had kindly agreed to assess the merit of our microwave assisted method against the classical reflux method. Three g of SRM 8436 Durum Wheat Flour was sent to each of these laboratories and the results were compared by linear regression analysis.

2.4.2 Optimization of microwave-assisted hydrolysis conditions

There are two methods that were optimized to gain high recovery of amino acids; the first one is to recover the non-sulfur containing amino acids (which is called non-oxidized method) and the second one was to recover the sulfur containing amino acids (which is called oxidized method). The non-oxidized and oxidized methods should be applied to each sample to recover both non-sulfur containing and sulfur-containing amino acids. As mentioned earlier, large amounts of methionine and cystine are lost due to degradation in the regular hydrolysis

method. Oxidation of those amino acids allows an accurate quantification of methionine as methionine sulphone (Met sln) and cystine as cysteic acid (Cyst acid). However, as this method causes a major loss of tyrosine, phenylalanine, histidine and arginine, it is typically used in parallel with the non-oxidizing methods to specifically quantify the sulfur-containing amino acids.

2.4.2.1 Optimization of the non-oxidized method

For optimization of microwave-assisted non-oxidized hydrolysis of wheat flour *T. durum* cv. Balcali 2000 was used as a model genotype. For this purpose, three variables were evaluated (i.e. sample mass, hydrolysis period and temperature) in three different experiments. For each variable tested, the obtained experimental results were presented along with the results obtained in the optimal conditions where 200 mg of wheat flour was hydrolyzed for 3 h at 150^{o}C.

The closed-vessel microwave system used in the acid hydrolysis tests was purchased from CEM Co., USA (Model: MarsExpress). The system was capable of processing up to 40 samples simultaneously by utilizing infrared sensors for temperature monitoring of individual vessels. Whole meal wheat flour samples (i.e. 100, 200, 300, 400 or 500 mg [±1 mg]) were hydrolyzed with 5 ml of 6 N HCl in 55 ml Teflon vessels with self-regulating pressure control. Vessels were capped immediately after purging the acid-sample suspension with N_2 for one minute to maintain an O_2 free environment and prevent oxidation of amino acids during hydrolysis. The microwave systems was programmed for ramping to the desired temperature (i.e. 130, 150 or 170^{o}C, [±5oC]) within 30 min and then stay constant for a given period of time (i.e. 1, 2, 3 or 4 h). Upon completion of the hydrolysis period, vessels were cooled down to room temperature in a water bath. The resulting hydrolysates were added to 10 ml with 6 N HCl and spikes of 1 ml was added with 550 µl of 32 % NaOH (for adjusting the pH to around 2.2, [±0.2]) and 5 ml of sodium citrate loading buffer (pH 2.2) in a total volume of 6.55 ml. Finally the samples were filtered through 0.22 µm syringe-tip filters in to 2 ml glass vials and stored at +4^{o}C until analysis.

Amino acids were analyzed by an automated amino acid analyzer (Biochrom 32 Oxidized Hydrolysate System, Biochrom Co., Cambridge, UK) with post column ninhydrin

derivatization. Sample injection volume was 20 µL for standards and samples. All essential amino acids were quantified except methionine, cysteine and tryptophan, which were destroyed during hydrolysis, asparagine and glutamine, which were converted to aspartic acid and glutamic acid respectively. In this method, the following amino acids were quantified: Aspartic acid (Asp), threonine (Thr), serine (Ser), glutamic acid (Glu), proline (Pro), glycine (Gly), alanine (Ala), valine (Val), isoleucine (Ile), leucine (Leu), tyrosine (Tyr), phenylalanine (Phe), histidine (His), lysine (Lys) and arginine (Arg).

2.4.2.2 Optimization of the oxidized method

Optimization of the oxidized method was conducted by three different experiments to evaluate the effect of three variables (i.e. sample mass, HCl volume and concentration) on the recovery of sulfur-containing amino acids. In the oxidized method, SRM 8436 Durum Wheat Flour (National Institute of Standards and Technology, Gaithersburg, USA) was initially subjected to an oxidation process by incubating the samples in performic acid at 0^{o}C for 16 h prior to the hydrolysis treatments. For every 10-20 mg of protein (or to 25-200 mg of wheat flour), 5 ml of performic acid was added. Prior to hydrolysis, any residual performic acid was quenched by the addition of 0.84 g sodium metabisulphite in each sample.

Following the oxidation of flour samples of different masses (i.e. 25, 50, 100 or 200 mg) the samples were then hydrolyzed in 5 ml 6 N, 10 ml 6 N and 5 ml 12 N HCl for 3 hours at 150^{o}C in a closed-vessel microwave system (CEM Co., USA, Model: MarsExpress). After adding all hydrolysates to 10 ml with 6 N HCl, sample preparation for amino acid analysis was finalized by spiking of a 1 ml hydrolysate, adjusting the pH to around 2.2 (± 0.2) and adding the loading buffer as follows: (i) for 5 ml 6 N HCl hydrolysates, 1 ml of hydrolysate was added with 5.175 ml sodium citrate loading buffer and 375 µl of 32% NaOH, (ii) for 10 ml 6 N HCl hydrolysates, 1 ml of hydrolysate was added with 5.1 ml sodium citrate loading buffer and 450 µl of 32% NaOH, (iii) for 5 ml 12 N HCl hydrolysates, 1 ml of hydrolysate was added with 4.9 ml sodium citrate loading buffer, 630 µl of 32% NaOH and 20 µl of 6 N HCl. The resulting oxidized hydrolysates were then quantified for cysteic acid and methionine sulphone with Biochrom 30 amino acid analyzer as described in section 2.4.2.1. Additionally, SRM 8436 Durum Wheat Flour was analyzed for cysteic acid and methionine

sulphone by two independent laboratories (i.e. Biochrom Co. and Ansynth Service B.V. laboratories).

2.4.2.3 Standard preparation

Mixed amino acids standard was used the same way all over this project and it was prepared as the following:

The total amino acids standard was prepared by adding the following volumes together:

- 1 volume of AAS 18(sigma std) concentration=2.5 µ mole/ml.
- 1 volume of methionine sulphone, concentration= 2.5 µ mole/ml.
- 1 volume of methionine suphoxide, concentration= 2.5 µ mole/ml.
- 1 volume of cysteic acid, concentration =2.5 µ mole/ml.
- 1 volume of ornithine, concentration= 2.5 µ mole/ml.
- 5 volumes of sodium citrate loading buffer with pH=2.2

Final concentration for all amino acids = 5 nmole 20 μl^{-1}, except cystine= 2.5 nmole 20 μl^{-1}.

- Methionine sulphone (2.5 µ mole/ml) preparation:

=2.5 x 181.21(MW) x 10^{-6} ml^{-1} =0.000453 g ml^{-1} or 0.0453 g in 5ml loading buffer, then take 50 µl from the mixture and add it to 950µl loading buffer.

- L-Ornithine (2.5 µ mole ml^{-1}) preparation:

2.5x168.62(MW)x10E^{-6}= 0.000422 g ml^{-1} loading buffer or 0.0422 in 5 ml buffer, then take 50 µl from the mixture and add it to 950 µl loading buffer.

- Methionine sulphoxide (2.5 µ mol ml^{-1}) preparation:

2.5x165.21 (MW)x10E^{-6}=0.000413 g ml^{-1} loading buffer, or 0.0413 g in 5 ml loading buffer, then take 50 µl from the mixture and add it to 950 µl loading buffer.

- Cystic acid (2.5µ mole ml^{-1}) preparation:

2.5x187.13(MW)x10E^{-6}= 0.000468 g ml $^{-1}$loading buffer, or o.o468 g in 5 ml loading buffer, then take 50 µl from the mixture and add it to 950 µl loading buffer.

2.4.2.4 Statistical analysis of results

In the optimization experiments, each treatment consisted of three independent replications. Analysis of variance procedure in JMP statistical package (version 5.0.1a, SAS Institute Inc., Cary, NC, 1989–2002) was used to test for treatment effects. Means were separated by Student's least significant difference (LSD) test when a significant ($P<0.05$) difference occurred.

2.4.2.5.Calculations

In order to have the final result of each amino acid and their total in units of g/100 g WF, the calculations were conducted as follows:

- Weight of sample used = W
- Total volume of hydrolysate= V
- Injection volume= 20 µl
- Amount of hydrolysate used + Buffer+ NaOH = 1000+5000+550=6550 µl
- 1 mole = 1000000000 n moles
- The result for each amino acid that is obtained from the AA analyzer in nano mole= X
- Molecular weight of each amino acid =M
- Amount of amino acid(in grams) for any amino acid found in 100 g wheat flour (100000 mg) =F
- $F=10^{-9}$ x M x X x 100000 / (W x 20/(10 x 6550))

Example: if the molecular weight for specific amino acid is 150, result obtained from the AA analyzer is 5, total volume of hydrolysate is 10, then F= 1.22 g/100 g WF for that specific amino acid.

3 RESULTS

3.1 Results of non-oxidized hydrolysis method

3.1.1 Optimization of temperature

Performing the hydrolysis at different temperatures (i.e. 130, 150 or 170^{o}C) affected the recovery rates of individual amino acids (Table 3-1). Most of the amino acids showed highest recovery at the 150^{o}C hydrolysis temperature, however the recovered concentration values for Arg, Glu, Gly, His, Pro, Thr and Tyr were not statistically different following hydrolysis at 130, 150 or 170^{o}C. Nevertheless, the absolute concentrations of 12 out of 16 individual amino acids were higher and accordingly the sum of all amino acids was significantly higher at 150^{o}C (Table 3-1). Under the given conditions 150^{o}C is suggested to be the optimum hydrolysis temperature for majority of the amino acids in wheat flour with a few exceptions. Increasing the hydrolysis temperature from 150 to 170^{o}C positively affected liberation of Val and Ile and significantly increased Leu concentration. On the contrary, concentration of Asp was severely reduced at 170^{o}C. Reduction of hydrolysis temperature to 130^{o}C resulted in decline of concentration values for all amino acids except Tyr. Although not statistically significant, recovery of Tyr was increased with reduction in hydrolysis temperature from 170 to 130^{o}C.

Table 3-1Amino acid concentration (g/100 g of whole wheat flour) as influenced by hydrolysis temperature

Amino acid	130 °C	150 °C	170 °C
Ala	0.42[a]±0.01[b] B[c]fg[d]	0.44±0.02 Ah	0.43±0.01 ABfg
Arg	0.44±0.01 Af	0.54±0.03 Aef	0.53±0.06 A de
Asp	0.53±0.04 Ade	0.59±0.02 Ae	0.31±0.04 Bhi
Glu	3.34±0.07 Aa	3.70±0.13 Aa	3.47±0.19 Aa
Gly	0.45±0.03 Aef	0.48±0.02 Agh	0.45±0.04 Af
His	0.29±0.02 Ahij	0.32±0.01 Ai	0.31±0.04 Ahi
Ile	0.27±0.06 Bj	0.36±0.05 ABi	0.45±0.03 Af
Leu	0.64±0.04 Cc	0.72±0.02 Bc	0.80±0.02 Ac
Lys	0.29±0.00 Bij	0.35±0.02 Ai	0.34±0.03 ABhi
Phe	0.35±0.06 Bjhi	0.47±0.03 Agh	0.37±0.02 Bgh
Pro	1.03±0.03 Ab	1.16±0.06 Ab	1.15±0.10 Ab
Ser	0.59±0.05 Acd	0.65±0.01 Ad	0.47±0.08 Bef
Thr	0.30±0.02 Ahij	0.36±0.02 Ai	0.31±0.04 Ahi
Tyr	0.37±0.09 Afgh	0.33±0.01 Ai	0.28±0.03 Ai
Val	0.34±0.10 Bghij	0.52±0.06 Afg	0.59±0.03 Ad
Total[e]	9.65 C	11.00 A	10.27 B
Recovery[f] (%)	75.0	85.5	79.8

[a]Mean value and [b] standard deviation of three independent analysis results. [c] Lack of capital letters in common indicate differences ($p < 0.05$) between hydrolysis temperatures within a given amino acid and [d] lack of small letters in common indicate differences ($p < 0.05$) between amino acids within a given hydrolysis temperature (Fisher's LSD).

3.1.2 Optimization of hydrolysis period

Among the variables investigated to find the optimum hydrolysis conditions, changing the hydrolysis period was the most effective on the liberation of amino acids from the wheat flour matrix. The total recovery was highest (i.e. 85.5 %) in the 3 h hydrolysis treatment (Table 3-2).Consequently, concentrations of all individual amino acids (except Pro) were significantly higher in the 3 h hydrolysis treatment. Reducing (i.e. 1 and 2 h) or prolonging the hydrolysis time (i.e. 4 h) resulted in significant reductions in the recovery rates of amino acids, revealing that 3 h was the optimum period of time for microwave-assisted hydrolysis of wheat flour (Table 3-2). The second most abundant amino acid Pro in the wheat flour exhibited an exceptional insensitivity to hydrolysis time. Within 2 to 4 h of hydrolysis period,

there was no significant change in Pro concentration (Table 3-2); however at 1 h hydrolysis treatment Pro concentration and thus recovery rate was reduced significantly. In contrast, the most abundant Glu, together with Ala and Gly concentrations were highly affected by the duration of hydrolysis (Table 3-2). A similar and significant reduction was detected in the recovery rates of individual amino acids at the 2 or 4 h hydrolysis treatments compared to the optimum period of 3 h. Obviously, 1 h was totally insufficient for the hydrolysis of wheat flour matrix, particularly for Phe, Ser, Tyr and Thr and concentrations of these amino acids were 3-3.5 fold higher at the optimum hydrolysis period of 3 h (Table 3-2).

Table 3-2: Amino Acid Concentration (g/100 g of Whole Wheat Flour) as Influenced by Hydrolysis Period

Amino acid	1 h	2 h	3 h	4 h
Ala	0.20[a]±0.02[b] D[c]de[d]	0.28±0.01 Bgh	0.44±0.02 Ah	0.26±0.00 Cg
Arg	0.20±0.01 Cdef	0.34±0.02 Bef	0.54±0.03 Aef	0.32±0.01 Bef
Asp	0.21±0.03 Cde	0.37±0.01 Bde	0.59±0.02 Aef	0.34±0.00 Be
Glu	1.85±0.09 Da	2.67±0.08 Ba	3.70±0.13 Aa	2.43±0.02 Ca
Gly	0.18±0.02 Ddefg	0.31±0.01 Bfg	0.48±0.02 Agh	0.27±0.00 Cg
His	0.13±0.02 Cfgh	0.21±0.00 Bi	0.32±0.01 Ai	0.20±0.00 Bhi
Ile	0.17±0.01 Befgh	0.19±0.02 Bi	0.36±0.05 Ai	0.21±0.02 Bh
Leu	0.34±0.03 Cc	0.53±0.02 Bc	0.72±0.02 Ac	0.51±0.01 Bc
Lys	0.14±0.01 Cefgh	0.21±0.01 Bi	0.35±0.02 Ai	0.20±0.00 Bh
Phe	0.13±0.02 Cfgh	0.26±0.04 Bh	0.47±0.03 Agh	0.30±0.04 Bf
Pro	0.94±0.10 Bb	1.16±0.01 Ab	1.16±0.06 Ab	1.06±0.03 ABb
Ser	0.19±0.06 Cdef	0.41±0.01 Bd	0.65±0.01 Ad	0.37±0.01 Bd
Thr	0.12±0.01 Cgh	0.22±0.01 Bi	0.36±0.02 Ai	0.21±0.00 Bh
Tyr	0.10±0.01 Ch	0.19±0.01 Bi	0.33±0.01 Ai	0.17±0.00 Bi
Val	0.25±0.02 Bd	0.29±0.03 Bgh	0.52±0.06 Afg	0.30±0.02 Bf
Total[e]	5.14 C	7.66 B	11.00 A	7.16 B
Recovery[f] (%)	40.9	59.5	85.5	55.6

[a] Mean value and [b] standard deviation of three independent analysis results. [c] Lack of capital letters in common indicate differences ($p < 0.05$) between hydrolysis periods within a given amino acid and [d] lack of small letters in common indicate differences ($p < 0.05$) between amino acids within a given hydrolysis period (Fisher's LSD).

3.1.3 Optimization of sample mass

Among the range of sample masses evaluated (100-500 mg), it was clearly shown that 200 mg was the optimum by yielding the highest recovery rate. With the exception of Tyr, His and Leu, liberation of all amino acids analyzed were significantly higher when a sample mass of 200 mg was used in the given hydrolysis conditions (Table 3-3). Variations in sample mass in the range of 100 to 500 mg had no significant effect on recovery of Leu. In the case of Tyr, 100 mg sample mass yielded the highest recovery and the recovery rate reduced with the increase in sample mass. In general, the values determined for individual amino acids were enhanced by increasing the sample mass from 100 to 200 mg. However, increasing the sample mass over 200 mg and up to 500 mg favored incomplete hydrolysis conditions, particularly for His, Tyr, Phe, Ser and Thr. At the 500 mg sample level, the concentration values and thus recovery rates were significantly reduced for all amino acids analyzed (Table 3-3). In the optimum sample mass conditions of 200 mg, Glu, Pro and Leu were the most abundant amino acids in the wheat flour whereas lowest concentrations were determined for Lys, Tyr and His (Table 3-3).

Table 3-3:. Amino Acid Concentration (g/100 g of Whole Wheat Flour) as Influenced by Sample Mass

Amino acid	100 mg	200 mg	300 mg	400 mg	500 mg
Ala	0.40[a]±0.02[b] B[c]fg[d]	0.44±0.02 Ah	0.41±0.03 Bdef	0.41±0.02 Bef	0.38±0.01 Bef
Arg	0.45±0.03 BCef	0.54±0.03 Aef	0.46±0.06 Bdef	0.42±0.01 BCef	0.38±0.02 Cef
Asp	0.55±0.02 Ad	0.59±0.02 Aef	0.55±0.05 Acde	0.52±0.04 ABd	0.46±0.06 Bde
Glu	3.21±0.19 ABa	3.70±0.13 Aa	3.12±0.53 Ba	3.22±0.12 ABa	2.87±0.14 Ba
Gly	0.46±0.01 ABef	0.48±0.02 Agh	0.39±0.11 ABdef	0.43±0.03 ABe	0.38±0.04 Bef
His	0.34±0.01 Agh	0.32±0.01 ABi	0.30±0.02 Bf	0.13±0.04 Ci	0.16±0.02 Cj
Ile	0.18±0.02 Ci	0.36±0.05 Ai	0.27±0.05 Bf	0.28±0.02 Bh	0.26±0.05 Bghi
Leu	0.66±0.04 Ac	0.72±0.02 Ac	0.70±0.15 Abc	0.77±0.03 Ac	0.70±0.04 Ac
Lys	0.32±0.02 ABh	0.35±0.02 Ai	0.26±0.07 BCf	0.27±0.01 BCh	0.24±0.01 Cij
Phe	0.48±0.02 Ae	0.47±0.03 Agh	0.35±0.05 Bef	0.36±0.01 Bfg	0.34±0.01 Bfgh
Pro	0.95±0.06 Bb	1.16±0.06 Ab	0.90±0.21 Bb	0.95±0.03 Bb	0.87±0.05 Bb
Ser	0.61±0.03 ABcd	0.65±0.01 Ad	0.61±0.05 Acd	0.58±0.05 ABd	0.49±0.11 Bd
Thr	0.28±0.02 Bh	0.36±0.02 Ai	0.31±0.05 ABef	0.30±0.01 Bgh	0.25±0.03 Bhi
Tyr	0.58±0.01 Ad	0.33±0.01 Bi	0.33±0.01 Bef	0.28±0.04 Bh	0.21±0.05 Cij
Val	0.34±0.01 Bgh	0.52±0.06 Afg	0.39±0.11 Bdef	0.36±0.04 Befg	0.35±0.07 Bfg
Total[e]	9.80 B	11.00 A	9.34 B	9.27 B	8.34 C
Recovery[f] (%)	76.1	85.5	72.6	72.0	64.8

[a] Mean value and [b] standard deviation of three independent analysis results. [c] Lack of capital letters in common indicate differences ($p < 0.05$) between hydrolysis periods within a given amino acid and [d] lack of small letters in common indicate differences ($p < 0.05$) between amino acids within a given hydrolysis period (Fisher's LSD).

3.2 Results of oxidized hydrolysis method

As shown in Table 3-4 the recovery of cysteic acid (Cyst acid) was highest when 10 ml 6 N HCl was used to hydrolyze either 25 mg or 50 mg of flour sample. Expressed in a decreasing order, the next highest recovery corresponded to 50 mg sample hydrolyzed in 5ml 6 N HCl followed by 100 mg samples in 10 ml 6 N HCl, 25mg samples in 5 ml 12 N HCl, 25 or 100 mg samples in 5 ml 6 N HCl, 200 mg sample in 10 ml 6 N HCl, 50mg sample in 5 ml 12 N HCl and lastly 200 mg samples in 5 ml 6 N HCl (Table 3-4).

The recovery of methionine sulphone (Met sln) was highest when 50 mg of flour sample was hydrolyzed with either 5 or 10 ml of 6N HCl. Additionally, 5ml 12 N HCl yielded the highest recovery rate when 25 mg of sample was used. Met sln recovery was significantly lower in treatments with 5 ml 6 N HCl to hydrolyze 25, 100 or 200 mg samples and 10 ml 6 N HCl to hydrolyze 25 or 200 mg sample, as well as 5 ml 12 N HCl to hydrolyze 50 or 200 mg sample. The lowest recovery for Met sln was detected when 100 mg of flour was hydrolyzed in 10 ml 6N HCl and 5 ml 12 N HCl (Table 3-4).

On the basis of the results above, the highest simultaneous recovery of both Cyst acid and Met sln was achieved by the use of 10 ml of 6N HCl and 50 mg of flour sample (Table 3-4).

Table 3-4:effects of different hcl volumes and concentrations and sample mass on hydrolysis of cysteic acid and methionine sulphone in NIST wheat flour.

HCl treatment	Mass treatment mg	Cyst acid (g/100 mg)	Met sln (g/100 mg)
5 ml 6 N	25	0.607[a]±0.033[b] CD[c]	0.233±0.040 AB
	50	0.719±0.012 AB	0.249±0.022 A
	100	0.636±0.016 CD	0.227±0.006 AB
	200	0.528±0.020 E	0.239±0.021 AB
10 ml 6 N	25	0.745±0.081 A	0.231±0.068 AB
	50	0.738±0.094 A	0.252±0.036 A
	100	0.655±0.010 BC	0.198±0.006 B
	200	0.610±0.006 CD	0.215±0.015 AB
5 ml 12 N	25	0.643±0.033 BC	0.246±0.003 A
	50	0.562±0.028 DE	0.235±0.004 AB
	100	0.301±0.011 G	0.118±0.012 C
	200	0.441±0.063 F	0.214±0.035 AB

[a] Mean value and [b] standard deviation of three independent analysis results. [c] Values in columns not sharing a common letter are significantly different.

3.3 Results of the round robin laboratory test of NIST wheat flour

In the round robin amino acid analysis, very similar results were found among the laboratories and the hydrolysis methods (i.e. 24 h reflux vs. 3 h microwave) (Table 3-5). For example results from the 3 h microwave hydrolysis (i.e. Sabanci University) for the most abundant amino acids Glu and Pro fell in the middle of three laboratories. In the case of Lys, the most limited amino acid in wheat flour, three labs had produced identical results. The recoveries of sulfur-containing amino acids were even higher when the oxidized method was combined with the microwave-assisted hydrolysis performed at the Sabanci University laboratory (Table 3-5). In addition very high correlations were found among the results of laboratories involved in the round robin testing of SRM 8436 Durum Wheat Flour (Figure 3.1).

Table 3-5: Amino acid analysis results from laboratories involved in the round robin testing of SRM 8436 Durum Wheat Flour (National Institute of Standards and Technology, Gaithersburg, USA). The same flour sample (SRM 8436 Durum Wheat Flour) was hydrolyzed either by the classical 24 h reflux method (i.e. Biochrom Co. and Ansynth Service B.V. laboratories) or by the optimized 3 h microwave-assisted method (i.e. Sabanci University).

Amino acid	Biochrom Co.	Sabanci Univ.	Ansynth B.V.
		(g/100g WF)	
Asp	0.50	0.49	0.52
Thr	0.34	0.33	0.36
Ser	0.62	0.63	0.59
Glu	4.55	4.66	4.73
Pro	1.45	1.46	1.51
Gly	0.46	0.45	0.47
Ala	0.38	0.38	0.38
Cyst(e)ine	0.68	0.74	0.29
Val	0.57	0.46	0.55
Methionine	0.19	0.25	0.21
Ileu	0.45	0.37	0.48
Leu	0.89	0.85	0.95
Tyr	0.38	0.30	0.41
Phe	0.59	0.66	0.64
His	0.26	0.28	0.26
Lys	0.24	0.24	0.24
Arg	0.46	0.41	0.48
TOTAL	**13.00**	**12.95**	**13.07**

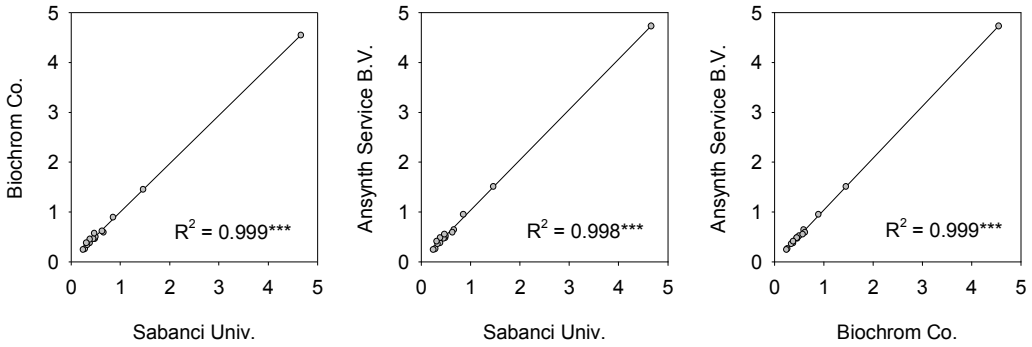

Figure 3-1: Correlations among the results of amino acid analysis from laboratories involved in the round robin testing of SRM 8436 Durum Wheat Flour (National Institute of Standards and Technology, Gaithersburg, USA). The same flour sample (SRM 8436 Durum Wheat Flour) was hydrolyzed either by the classical 24 h reflux method (i.e. Biochrom Co. and Ansynth Service B.V. laboratories) or by the optimized 3 h microwave-assisted method (i.e. Sabanci University).

3.4 Amino acid profiles and their correlations with mineral nutrients in modern, primitive and wild wheat genotypes

3.4.1 Analysis of modern wheat genotypes

Nineteen modern wheat genotypes were evaluated for the concentrations of Cyst Acid, Meth sln, Asp, Thr, Ser, Glu, Pro, Gly, Ala, Val, Ile, Leu, Tyr, Phe, His, Lys and Arg. The modern wheat genotypes were grouped into two as nine bread and ten durum genotypes. Each amino acid and the sum of the amino acids have been investigated to find out the possible correlations with the mineral nutrients (i.e. N, K, P, Mg, S, Ca, Fe, Mn, Cu and Zn) analyzed in the whole meal flour of the genotypes.

Among the amino acids analyzed concentration of Glu was the highest in both bread and durum wheat genotypes (Tables 3.6 and 3.7). In bread wheat genotypes, Glu ranged between 3.31 and 4.80 g/100g wf and in average constituted about 34% of the total amino acids. In durum wheat genotypes Glu ranged between 2.49 and 4.20 g/100g wf and in average constituted 33% of the total amino acids. The second highest amino acid was Pro and ranged between 0.98 and 1.53 g/100 g wf in bread and 0.79 and 1.25 g/100 g wf in durum wheat genotypes (Tables 3.6 and 3.7). The share of Pro in total amino acid concentration was 11% in bread and 10% in durum wheat genotypes. In bread wheat genotypes, Met sln had the lowest amino acid concentration ranging between 0.193-0.319 g/100 g wf, whereas Met sln was the second lowest amino acid in the durum wheat genotypes. In durum wheat genotypes Ile had the lowest concentration ranging between 0.132-3.55 g/100 g wf.

When the variations among the genotypes are investigated, it can be seen that Cyst acid was highly stable among both bread and durum wheat genotypes with only a little variation (% cv: coefficient of variation) of about 8-9 % (Tables 3.6 and 3.7). Among the bread wheat genotypes, Ser and Thr expressed the highest variation. For example Ser and Thr was 3.3 and 2.3 fold higher in Karahan compared to Bezostaja. The total amino acid content ranged between 9.7 g/100 g wf (Alpu 1) and 14.8 g/100 g wf (Karahan) with an average of 10.7 in the bread wheat genotypes. Among the durum wheat genotypes total amino acid content ranged between 8.3 g/100 g wf (Gediz) and 11.8 g/100 g wf (Selcuklu) with an average of 10.0. The coefficient of variation for the total amino acid content was calculated as 14 % for both bread and durum wheat genotypes (Tables 3.6 and 3.7).

Table 3-6:Concentration of amino acids in modern bread wheat genotypes.

Genotype	Cyst acid	Met sln	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Val	Ile	Leu	Tyr	Phe	His	Lys	Arg	Total
									g/100 g wf									
Karahan	0.753	0.319	0.737	0.418	0.741	4.80	1.53	0.610	0.527	0.529	0.403	0.958	0.367	0.716	0.369	0.398	0.636	14.8
Adana 99	0.666	0.217	0.633	0.362	0.640	3.89	1.15	0.483	0.437	0.458	0.339	0.758	0.284	0.565	0.276	0.319	0.498	11.4
Ahmetaga	0.703	0.235	0.608	0.282	0.616	3.83	1.23	0.553	0.442	0.329	0.256	0.717	0.398	0.604	0.366	0.298	0.467	11.7
Alpu 1	0.633	0.193	0.482	0.187	0.248	3.31	0.98	0.414	0.410	0.424	0.311	0.642	0.231	0.484	0.240	0.291	0.429	9.7
Gerek	0.688	0.245	0.655	0.352	0.643	4.17	1.34	0.524	0.445	0.452	0.341	0.820	0.330	0.644	0.319	0.349	0.531	12.7
Bezostaja	0.639	0.250	0.421	0.181	0.225	3.95	1.21	0.483	0.445	0.529	0.381	0.766	0.255	0.561	0.271	0.305	0.667	10.9
Tosun bey	0.762	0.259	0.639	0.363	0.677	4.47	1.41	0.568	0.478	0.435	0.314	0.821	0.416	0.681	0.381	0.322	0.482	13.1
Kirgiz	0.686	0.204	0.556	0.291	0.552	3.51	1.19	0.438	0.380	0.373	0.261	0.688	0.292	0.581	0.273	0.285	0.383	11.0
C-1252	0.738	0.246	0.528	0.285	0.598	4.03	1.40	0.456	0.380	0.309	0.254	0.726	0.408	0.594	0.349	0.254	0.427	11.7
mean	0.627	0.217	0.526	0.272	0.494	3.59	1.15	0.453	0.394	0.384	0.286	0.690	0.298	0.543	0.284	0.282	0.452	10.7
stdev	0.047	0.037	0.097	0.081	0.185	0.45	0.16	0.065	0.046	0.078	0.054	0.092	0.069	0.069	0.052	0.041	0.095	1.5
cv (%)	8	17	18	30	37	13	14	14	12	20	19	13	23	13	18	15	21	14

Table 3-7: Concentration of amino acids in modern durum wheat genotypes

Genotype	Cyst acid	Met sln	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Val	Ile	Leu	Tyr	Phe	His	Lys	Arg	Total
									g/100 g wf									
Tuten	0.572	0.236	0.458	0.199	0.453	2.60	0.80	0.351	0.320	0.164	0.149	0.553	0.336	0.516	0.192	0.220	0.316	8.4
Gediz	0.686	0.204	0.456	0.194	0.420	2.49	0.79	0.349	0.318	0.143	0.149	0.521	0.330	0.478	0.251	0.216	0.324	8.3
EGE 2005	0.615	0.271	0.487	0.222	0.470	2.84	0.91	0.382	0.358	0.221	0.168	0.559	0.338	0.513	0.274	0.237	0.328	9.1
Zenit	0.623	0.187	0.582	0.278	0.583	3.82	1.21	0.449	0.427	0.173	0.214	0.746	0.408	0.654	0.315	0.274	0.394	11.1
Meram	0.591	0.275	0.549	0.304	0.604	3.54	1.14	0.421	0.392	0.387	0.320	0.674	0.305	0.624	0.298	0.301	0.471	9.3
Yelken	0.594	0.259	0.495	0.242	0.516	3.40	1.08	0.397	0.357	0.285	0.198	0.599	0.309	0.542	0.276	0.260	0.379	10.2
Kumbet	0.664	0.223	0.409	0.186	0.456	2.66	0.83	0.372	0.310	0.192	0.132	0.525	0.329	0.489	0.277	0.203	0.290	8.5
Selcuklu	0.555	0.254	0.600	0.323	0.593	3.84	1.23	0.482	0.409	0.431	0.313	0.782	0.307	0.617	0.305	0.341	0.505	11.8
Yilmaz	0.714	0.252	0.573	0.239	0.580	4.20	1.25	0.428	0.439	0.290	0.330	0.731	0.407	0.643	0.317	0.271	0.408	11.5
Balcali 2000	0.726	0.230	0.535	0.332	0.571	3.61	1.18	0.425	0.406	0.151	0.355	0.805	0.291	0.608	0.292	0.314	0.502	11.3
mean	0.634	0.239	0.514	0.252	0.525	3.30	1.04	0.406	0.374	0.244	0.233	0.650	0.336	0.568	0.280	0.264	0.392	10.0
stdev	0.060	0.029	0.063	0.054	0.069	0.61	0.19	0.043	0.047	0.102	0.087	0.111	0.041	0.067	0.037	0.046	0.079	1.4
cv (%)	9	12	12	22	13	18	18	11	13	42	37	17	12	12	13	17	20	14

Among the macro nutrients analyzed, concentrations of Ca (range: 0.020-0.049 % in bread genotypes and 0.0 21-0.040 % in durum genotypes) and P (range: 0.215-0.368 % in bread genotypes and 0.235-0.366 % in durum genotypes) showed the largest variation (Tables 3.8 and 3.9). There was no significant variation in the N, K, Mg or S concentrations within bread or durum wheat genotypes; however average K and P was %19 higher in durum wheat genotypes compared to bread wheat (Tables 3.8 and 3.9). Focusing on the micronutrients, concentration Fe and Mn were found similar within and among the bread or durum wheat genotypes, however Cu (range: 3.69-6.10 mg kg^{-1} in bread genotypes and 3.74-8.76 mg kg^{-1} in durum genotypes) and Zn (range: 11.4-21.3 mg kg^{-1} in bread genotypes and 9.9-36.0 mg

kg^{-1} in durum genotypes) showed a higher variation, particularly in the durum wheat genotypes (Tables 3.8 and 3.9). In addition to the higher, average concentrations of Cu and Zn was also %28 higher in durum wheat compared to bread wheat genotypes (Tables 3.8 and 3.9).

Table 3-8 : Concentration of mineral nutreints in modern bread wheat genotypes

Genotype	N	K	P	Mg	S	Ca	Fe	Mn	Cu	Zn
			%					mg kg^{-1}		
Karahan	2.69	0.341	0.221	0.117	0.172	0.039	30.9	30.5	4.39	20.4
Adana 99	2.28	0.409	0.359	0.131	0.146	0.049	41.8	45.2	4.45	19.4
Ahmetaga	2.60	0.396	0.368	0.150	0.158	0.042	48.1	44.0	5.29	16.0
Alpu 1	2.12	0.386	0.326	0.132	0.139	0.039	35.0	35.1	3.95	17.6
Gerek	2.48	0.374	0.215	0.134	0.153	0.039	35.6	35.3	3.76	21.3
Bezostaja	2.41	0.354	0.232	0.101	0.145	0.030	35.5	32.6	3.69	18.4
Tosun bey	2.81	0.342	0.270	0.132	0.167	0.027	34.9	31.4	3.99	16.9
Kirgiz	2.24	0.347	0.278	0.131	0.149	0.031	32.5	35.2	3.69	11.4
C-1252	2.57	0.409	0.256	0.121	0.165	0.020	36.1	35.0	6.10	13.1
mean	2.22	0.336	0.253	0.115	0.139	0.032	33.0	32.4	3.93	15.5
stdev	0.23	0.028	0.058	0.014	0.011	0.009	5.2	5.2	0.83	3.3
cv (%)	10	8	23	12	8	28	16	16	21	21

Table 3-9: Concentration of mineral nutreints in modern durum wheat genotypes

Genotype	N	K	P	Mg	S	Ca	Fe	Mn	Cu	Zn
			%					mg kg^{-1}		
Tuten	1.85	0.438	0.339	0.108	0.116	0.032	30.9	32.8	4.22	20.5
Gediz	1.79	0.420	0.366	0.124	0.115	0.033	32.9	33.9	8.76	22.3
EGE 2005	2.07	0.399	0.356	0.121	0.129	0.032	35.5	29.0	4.66	23.2
Zenit	2.55	0.401	0.303	0.123	0.154	0.026	31.5	32.4	7.52	36.0
Meram	2.07	0.445	0.274	0.122	0.139	0.035	33.7	34.4	5.93	25.7
Yelken	2.16	0.349	0.235	0.100	0.123	0.022	29.9	24.9	4.21	10.0
Kumbet	1.91	0.380	0.267	0.113	0.127	0.021	33.1	30.0	3.74	9.9
Selcuklu	2.19	0.426	0.265	0.117	0.129	0.029	28.8	30.2	5.07	24.4
Yilmaz	2.76	0.454	0.364	0.136	0.161	0.028	44.4	30.4	5.27	16.0
Balcali 2000	2.21	0.431	0.337	0.109	0.137	0.040	35.0	43.3	4.25	28.2
mean	2.16	0.414	0.311	0.117	0.133	0.030	33.6	32.1	5.36	21.6
stdev	0.30	0.032	0.048	0.010	0.015	0.006	4.4	4.8	1.62	8.0
cv (%)	14	8	15	9	11	19	13	15	30	37

Correlations in individual amino acids of bread wheat genotypes are provided in table 3.10. Among the bread wheat genotypes there existed a highly significant correlation (P<0.001) between Cyst acid, Tyr and His concentrations. Similarly, a high correlation was also evident

among Met sln, Pro and Glu and among Asp, Thr and Ser (P<0.001). Besides Pro and Met sln, Glu also significantly correlated with Leu and Phe. Phe was interesting because it had the highest number of correlations with other amino acids. Phe correlated well with 13 out of 16 amino acids analyzed. Among the amino acids present in the wheat flour Val, Ile and Arg had the fewest number of correlations with other amino acids including Phe. For example Val and Arg had only one significant correlation each, they both correlated significantly with Ile only (P<0.001). In the bread wheat genotypes, sum of all amino acids (without Trp) correlated well with 14 out of 17 amino acids, but did not correlate significantly with Arg, Ile and Val.

Table 3-10: Correlation among amino acids in bread modern genotypes

	Met sln	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Val	Ile	Leu	Tyr	Phe	His	Lys	Arg	sum w/o Trp
Cyst acid	0.670*	0.659	0.720*	0.804**	0.758*	0.876**	0.677*	0.410	-0.189	-0.099	0.599	0.912***	0.846**	0.913***	0.286	-0.030	0.803**
Met sln		0.559	0.578	0.486	0.944***	0.886***	0.838**	0.806**	0.462	0.583	0.930***	0.534	0.851**	0.712*	0.707*	0.698*	0.901***
Asp			0.956***	0.905***	0.683*	0.631	0.763**	0.638	0.146	0.227	0.720*	0.533	0.794*	0.629	0.737*	0.113	0.840**
Thr				0.939***	0.741*	0.710*	0.703*	0.568	0.160	0.235	0.749*	0.557	0.823**	0.621	0.665	0.117	0.854*
Ser					0.644	0.716*	0.659	0.382	-0.164	-0.074	0.586	0.745*	0.791*	0.743*	0.436	-0.090	0.783*
Glu						0.920***	0.872**	0.808**	0.433	0.539	0.954***	0.620	0.925***	0.769*	0.715*	0.596	0.953***
Pro							0.761*	0.560	0.133	0.250	0.832**	0.769*	0.934***	0.843**	0.505	0.371	0.920***
Gly								0.886***	0.337	0.420	0.852**	0.638	0.893***	0.817**	0.756*	0.529	0.899***
Ala									0.690*	0.745*	0.870**	0.269	0.715*	0.515	0.896***	0.726*	0.785*
Val										0.976***	0.610	-0.418	0.237	-0.184	0.736*	0.837**	0.326
Ile											0.703*	-0.310	0.315	-0.069	0.786*	0.891***	0.432
Leu												0.408	0.884**	0.601	0.873**	0.698*	0.942***
Tyr													0.722*	0.961***	0.078	-0.107	0.653
Phe														0.838*	0.681*	0.376	0.973***
His															0.316	0.126	0.794*
Lys																0.656	0.774*
Arg																	0.453

*,** and *** indicate significance at P≤0.05,0.01,0.001 respectively

Correlations between the amino acids and mineral nutrient concentrations in bread wheat genotypes are provided in table 3.11. Among the macro nutrients, N and S had significant positive correlations with Cyst acid, Met sln, Glu, Pro, Gly, Tyr, Phe, His and the sum of all amino acids (Table 3.11). There was no correlation present among amino acids and the micro nutrients Fe, Mn and Cu. However, Zn showed a significant correlation (P<0.05) with Ala, Val, Lys and Arg. Although not significantly correlated, amino acids expressed a negative relationship particularly with K, P, Fe and Mn concentrations (Table 3.11).

Table 3-11: Correlation between amino acids and nutrients in the bread modern genotypes

	N	K	P	Mg	S	Ca	Fe	Mn	Cu	Zn
Cyst acid	0.862**	-0.261	-0.291	0.124	0.875**	-0.386	-0.164	-0.315	0.415	-0.134
Met sln	0.790*	-0.451	-0.597	-0.390	0.773*	-0.136	-0.302	-0.493	0.130	0.415
Asp	0.529	-0.171	-0.081	0.376	0.474	0.383	-0.004	0.057	0.062	0.388
Thr	0.553	-0.188	-0.183	0.216	0.532	0.224	-0.111	-0.009	0.082	0.305
Ser	0.618	-0.031	-0.060	0.396	0.605	0.113	0.083	0.126	0.325	0.066
Glu	0.849**	-0.415	-0.552	-0.268	0.809**	-0.117	-0.265	-0.423	0.091	0.452
Pro	0.868**	-0.375	-0.623	-0.204	0.841**	-0.352	-0.304	-0.460	0.270	0.139
Gly	0.844**	-0.394	-0.244	0.083	0.705*	0.164	0.069	-0.160	0.054	0.474
Ala	0.609	-0.492	-0.277	-0.151	0.502	0.311	-0.122	-0.281	-0.225	0.712*
Val	0.019	-0.538	-0.441	-0.602	-0.069	0.295	-0.435	-0.398	-0.656	0.750*
Ile	0.113	-0.441	-0.480	-0.610	0.069	0.303	-0.401	-0.385	-0.498	0.815
Leu	0.683*	-0.493	-0.583	-0.312	0.619	0.075	-0.342	-0.417	-0.099	0.586
Tyr	0.884	-0.002	-0.107	0.303	0.875**	-0.358	0.188	-0.065	0.609	-0.184
Phe	0.860**	-0.503	-0.504	-0.016	0.736*	-0.122	-0.231	-0.373	0.051	0.263
His	0.958***	-0.153	-0.195	0.216	0.912***	-0.246	0.131	-0.154	0.481	0.027
Lys	0.370	-0.499	-0.386	-0.118	0.249	0.453	-0.286	-0.272	-0.386	0.765*
Arg	0.319	-0.385	-0.503	-0.653	0.247	0.156	-0.185	-0.329	-0.269	0.703*
Sum w/o Trp	0.804**	-0.423	-0.488	-0.062	0.740*	-0.001	-0.246	-0.347	0.088	0.395

*,** and *** indicate significance at P≤0.05,0.01,0.001 respectively

When the relationships among macro and micro nutrients are evaluated, it is found that N and S had the highest correlation (r=0.916, P<0.001) within the bread wheat genotypes. Following this, the second highest correlation was found in Fe and Mn (r=0.877, P<0.01). Grain Mn concentrations also correlated well with K and P (P<0.05) and also Fe correlated well with P (P<0.05). The remaining nutrients Mg, Ca and Cu had no correlation with the other nutrients.

Table 3-12: Correlation among nutrients in the bread modern genotypes

	K	P	Mg	S	Ca	Fe	Mn	Cu	Zn
N	-0.305	-0.346	0.004	0.916***	-0.331	0.020	-0.312	0.325	0.128
K		0.592	0.356	-0.067	0.270	0.661	0.747*	0.659	-0.068
P			0.644	-0.253	0.513	0.766*	0.834*	0.269	-0.203
Mg				-0.085	0.399	0.594	0.597	0.198	-0.147
S					-0.359	0.015	-0.248	0.583	0.057
Ca						0.456	0.638	-0.209	0.585
Fe							0.877**	0.451	-0.002
Mn								0.341	-0.024
Cu									-0.343

*,** and *** indicate significance at P≤0.05,0.01,0.001 respectively

Correlations among individual amino acids of durum wheat genotypes are provided in Table 3-13. There was no correlation between any of the amino acids with both Cyst acid and Tyr. Met sln and Val correlated with each other (P<0.05); however both Met sln and Val had no correlation with any other amino acids. On the contrary, Asp correlated well with all amino acids except Tyr and Val. Thr, Ser, Glu, Pro, Gly, Ala, Ile, Leu, Phe, His, Lys and Arg correlated well with each other with the exceptions of His - Lys and His - Arg. The sum of all amino acids correlated well with all the individual amino acids except Cyst acid, Met sln, Val and Tyr.

Table 3-13: Correlation among amino acids in durum wheat genotypes

	Met_sln	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Val	Ile	Leu	Tyr	Phe	His	Lys	Arg	sum_w_o_Trp
Cyst_acid	-0.353	-0.112	-0.087	-0.065	0.085	0.052	-0.144	0.137	-0.511	0.203	0.126	0.223	0.020	0.253	-0.144	-0.026	0.127
Met_sln		0.137	0.199	0.235	0.173	0.177	0.118	0.104	0.679*	0.334	0.013	-0.393	0.068	0.066	0.294	0.274	0.037
Asp			0.807**	0.908***	0.913***	0.926***	0.917***	0.946***	0.567	0.799**	0.895***	0.265	0.933***	0.703*	0.872***	0.818**	0.869***
Thr				0.872***	0.734*	0.823**	0.844**	0.754*	0.497	0.857**	0.898***	-0.267	0.788**	0.601	0.969***	0.970***	0.746*
Ser					0.934***	0.957***	0.912***	0.914***	0.614	0.879***	0.905***	0.112	0.963***	0.764**	0.891***	0.874***	0.823**
Glu						0.987***	0.889***	0.962***	0.537	0.836**	0.890***	0.313	0.945***	0.804**	0.800**	0.769**	0.926***
Pro							0.924***	0.957***	0.547	0.860***	0.924***	0.198	0.943***	0.820**	0.868***	0.838**	0.935***
Gly								0.873***	0.625	0.760*	0.902***	0.121	0.874***	0.799**	0.896***	0.828**	0.900***
Ala									0.423	0.830**	0.912***	0.382	0.960***	0.795**	0.792**	0.760*	0.903***
Val										0.532	0.375	-0.207	0.460	0.457	0.641	0.580	0.399
Ile											0.885***	-0.093	0.826**	0.641*	0.889***	0.931***	0.770**
Leu												0.098	0.911***	0.687*	0.905***	0.896***	0.923***
Tyr													0.334	0.267	-0.219	-0.278	0.229
Phe														0.728*	0.805**	0.785**	0.838**
His															0.621	0.592	0.743*
Lys																0.982***	0.811**
Arg																	0.761*

*,** and *** indicate significance at P≤0.05,0.01,0.001 respectively

Correlations between the amino acids and mineral nutrient concentrations in durum wheat genotypes are provided in Table 3-14. Among the macro nutrients, N correlated positively with Asp, Ser, Glu, Pro, Gly, Ala, Leu, Tyr, Phe, His and the sum of the amino acids. Also, S correlated positively well with Asp, Ser, Glu, Pro, Ala, Ile, Leu, Phe, His and the sum of the amino acids. There was no correlation found between any other nutrient with any of the amino acids except between Mg and Tyr and between Fe and Cyst acid. There was a tendency of a negative relationship between the P and amino acid concentration of the durum wheat genotypes (Table 3-14).

Table 3-14: Correlation between amino acids and nutrients in durum wheat genotypes

	N	K	P	Mg	S	Ca	Fe	Mn	Cu	Zn
Cyst_acid	0.271	0.190	0.539	0.346	0.328	0.235	0.683*	0.493	0.141	-0.047
Met_sln	-0.014	0.099	-0.206	-0.068	0.038	0.101	0.177	-0.291	-0.533	-0.341
Asp	0.754*	0.408	-0.075	0.392	0.659*	0.186	0.154	0.136	0.208	0.567
Thr	0.409	0.297	-0.273	-0.017	0.444	0.418	-0.105	0.457	-0.026	0.571
Ser	0.717*	0.341	-0.299	0.242	0.747*	0.138	0.164	0.211	-0.020	0.431
Glu	0.888***	0.277	-0.180	0.332	0.798**	-0.008	0.336	0.066	-0.005	0.290
Pro	0.828**	0.234	-0.236	0.252	0.744	0.051	0.235	0.126	-0.001	0.358
Gly	0.685*	0.196	-0.340	0.224	0.629	-0.012	0.003	0.079	0.012	0.440
Ala	0.891***	0.386	0.018	0.450	0.838**	0.162	0.386	0.198	0.114	0.499
Val	0.234	0.162	-0.526	0.144	0.211	-0.142	-0.061	-0.347	-0.153	-0.092
Ile	0.585	0.554	-0.033	0.262	0.639*	0.440	0.357	0.452	-0.079	0.330
Leu	0.703*	0.397	-0.083	0.194	0.686*	0.292	0.158	0.427	0.000	0.541
Tyr	0.672*	0.149	0.378	0.654*	0.585	-0.334	0.503	-0.242	0.356	0.177
Phe	0.821**	0.424	-0.118	0.368	0.827**	0.152	0.261	0.244	0.104	0.527
His	0.745*	0.024	-0.233	0.457	0.727*	-0.151	0.333	0.010	0.144	0.248
Lys	0.470	0.338	-0.277	0.058	0.446	0.333	-0.053	0.311	-0.040	0.464
Arg	0.424	0.406	-0.226	0.057	0.438	0.433	0.005	0.439	-0.024	0.450
sum_w_o_Trp	0.820**	0.170	-0.131	0.188	0.661*	0.007	0.206	0.121	-0.056	0.320

*,** and *** indicate significance at P≤0.05,0.01,0.001 respectively

Evaluation of the relationships among nutrients in durum wheat genotypes (Table 3-15) have shown that that N and S had the highest correlation (r=0.885, $P<0.001$). The second highest correlation was found between Mn and Ca (r=0.820, $P<0.01$). Ca correlated well with K ($P<0.05$) and Fe also correlated well with both Mg and S ($P<0.05$). The remaining nutrients had no correlation with any of the nutrients.

Table 3-15: Correlation among nutrients in durum wheat genotypes

	K	P	Mg	S	Ca	Fe	Mn	Cu	Zn
N	0.199	0.076	0.499	0.885***	-0.157	0.559	-0.052	0.059	0.234
K		0.556	0.576	0.392	0.674*	0.464	0.562	0.206	0.415
P			0.535	0.111	0.554	0.596	0.398	0.321	0.276
Mg				0.562	0.112	0.683*	0.039	0.540	0.288
S					0.013	0.640*	0.185	-0.046	0.313
Ca						0.154	0.820**	0.169	0.566
Fe							0.129	-0.021	-0.129
Mn								0.135	0.565
Cu									0.516

*,** and *** indicate significance at P≤0.05,0.01,0.001 respectively

3.4.2 Analysis of spelt wheat genotypes

Nine spelt wheat genotypes were evaluated for the concentrations of Cyst Acid, Meth sln, Asp, Thr, Ser, Glu, Pro, Gly, Ala, Val, Ile, Leu, Tyr, Phe, His, Lys and Arg. Each amino acid and the sum of the amino acids have been investigated to find out the possible correlations with the mineral nutrients (i.e. N, K, P, Mg, S, Ca, Fe, Mn, Cu and Zn) analyzed in the whole meal flour of the spelt genotypes.

In spelt genotypes, Glu concentration was the highest among the amino acids analyzed (Table 3-16). The range of Glu was between 3.68 and 7.90 g/100 g wf and in average constituted about 34.5% of the total amino acids. The second highest amino acid was Pro and ranged between 0.83 and 2.48 g/100 g wf. Pro constituted about 10 % of the total amino acids concentration. Met sln had shown the lowest amino acid concentration ranging between 0.175 and 0.336 g/100 g wf and Thr had the second lowest amino acid concentration ranging between 0.14 and 0.613 g/ 100 g wf (Table 3-16).

Concerning the variations of amino acids among the spelt wheat genotypes, it is noteworthy to state that Cyst acid had the lowest variation (cv: 19 %). The highest variations were found with Ile (cv: 48%) and Pro (cv: 46%) (Table 3-16). For example the second most abundant amino acid Pro was up to three fold different among the spelt genotypes (i.e. Sp 244 vs. Sp 211) (Table 3-16). The total amino acid content ranged between 9.1 g/ 100 g wf (Sp 211) and 22.4 g/100g wf (Sp 244). The coefficient of variation for the total amino acid content was calculated as 34 % for the spelt wheat genotypes (Table 3-16).

Table 3-16: Concentration of amino acids in spelt wheat genotypes

Genotype	Cyst acid	Met sln	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Val	Ile	Leu	Tyr	Phe	His	Lys	Arg	Total
									g/100 g wf									
Sp 211	0.699	0.205	0.643	0.305	0.639	3.68	0.94	0.554	0.480	0.362	0.201	0.713	0.394	0.655	0.358	0.308	0.454	11.6
Sp 207	0.645	0.188	0.756	0.334	0.647	3.88	1.01	0.564	0.490	0.356	0.230	0.816	0.501	0.754	0.411	0.337	0.444	12.4
Sp 89	0.675	0.175	0.692	0.302	0.685	4.15	1.06	0.580	0.475	0.357	0.204	0.816	0.491	0.750	0.313	0.312	0.477	12.5
Sp 21	0.566	0.176	0.358	0.140	0.145	3.30	0.83	0.377	0.406	0.474	0.315	0.583	0.216	0.448	0.197	0.261	0.348	9.1
Sp 663	0.823	0.336	1.027	0.613	1.098	7.57	2.38	0.900	0.777	0.917	0.641	1.527	0.596	1.143	0.527	0.550	0.810	22.2
Sp 244	0.913	0.311	1.019	0.580	1.119	7.90	2.48	0.873	0.760	0.809	0.624	1.503	0.565	1.163	0.521	0.506	0.770	22.4
Sp 926	0.860	0.189	0.947	0.453	0.776	7.19	2.14	0.766	0.781	0.711	0.525	1.301	0.576	0.969	0.475	0.466	0.646	19.8
Sp 818	0.910	0.325	0.967	0.474	1.030	7.36	2.19	0.790	0.688	0.727	0.493	1.272	0.647	1.087	0.492	0.421	0.637	20.5
Sp 804	0.875	0.218	0.769	0.412	0.841	6.40	1.94	0.681	0.702	0.652	0.463	1.116	0.559	0.877	0.379	0.389	0.580	17.9
mean	0.697	0.212	0.718	0.361	0.698	5.14	1.50	0.609	0.556	0.537	0.370	0.965	0.455	0.785	0.367	0.355	0.517	14.8
stdev	0.129	0.068	0.219	0.149	0.302	1.91	0.68	0.172	0.152	0.214	0.177	0.351	0.130	0.242	0.109	0.099	0.156	5.1
cv (%)	19	32	31	41	43	37	46	28	27	40	48	36	29	31	30	28	30	34

Among the macro nutrients analyzed, concentrations of Ca (range: 0.020-0.06) showed the largest variation followed by N (range: 1.7-4.4 %) (Table 3-17). The lowest variation was found with K (range: 0.409-0.562). In regard of the micronutrients, concentration of Cu (range: 4.55-14.07 mg kg^{-1}) showed the highest variation and the lowest variation was found with Fe (range: 35.0-84.1 mg kg^{-1})

Table 3-17: Concentration of mineral nutrients in spelt wheat genotypes

Genotype	N	K	P	Mg	S	Ca	Fe	Mn	Cu	Zn
			%					mg kg^{-1}		
Sp 211	2.36	0.472	0.376	0.109	0.163	0.041	39.6	23.0	5.27	25.9
Sp 207	2.48	0.435	0.353	0.096	0.153	0.030	35.0	30.5	4.55	25.7
Sp 89	2.47	0.409	0.374	0.091	0.146	0.040	35.8	33.8	5.65	26.2
Sp 21	1.70	0.445	0.331	0.113	0.176	0.060	44.6	19.1	6.76	37.4
Sp 663	4.40	0.531	0.537	0.141	0.225	0.040	59.4	53.7	7.55	50.6
Sp 244	4.23	0.440	0.534	0.184	0.243	0.020	79.4	52.8	11.51	66.3
Sp 926	4.33	0.490	0.667	0.204	0.250	0.022	84.1	45.8	14.07	54.3
Sp 818	4.25	0.562	0.612	0.202	0.252	0.020	70.2	47.4	12.11	53.4
Sp 804	4.00	0.452	0.484	0.121	0.233	0.031	60.7	31.5	6.37	60.0
mean	3.02	0.424	0.427	0.126	0.184	0.031	50.9	33.8	7.38	40.0
stdev	1.08	0.049	0.122	0.045	0.044	0.013	18.8	12.8	3.45	15.8
cv (%)	36	12	29	36	24	42	37	38	47	40

Data were presented by Dr Ozturk at The Proceedings of the International Plant Nutrition Colloquium (Sacramento/Ca/USA, 26-30 Aug 2009)

Correlations among individual amino acids of spelt wheat genotypes are provided in Table 3-18. There was no correlation found between Cyst acid and Met sln. Also, there was no correlation found between Tyr and Met sln, Val and Ile. The remaining amino acids and the total amino acids correlated well with each other. Among all the amino acids, the highest correlations were found in Glu and Pro with the other amino acids, where as the lowest correlation was found with Met sln (Table 3-18).

Table 3-18: Correlations among amino acids in spelt wheat genotypes

	Met sln	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Val	Ile	Leu	Tyr	Phe	His	Lys	Arg	sum w/o Trp
Cyst acid	0.696	0.859**	0.852**	0.871**	0.941***	0.933***	0.888***	0.922***	0.798**	0.804*	0.885**	0.848**	0.895***	0.821**	0.807**	0.847**	0.931***
Met sln		0.740*	0.824**	0.812**	0.777*	0.788*	0.817**	0.674*	0.812**	0.769*	0.802**	0.619	0.828**	0.761*	0.777*	0.826**	0.809**
Asp			0.964***	0.952***	0.897***	0.886***	0.973***	0.889***	0.757*	0.754*	0.935***	0.933***	0.978	0.982***	0.919***	0.923***	0.935***
Thr				0.957***	0.919***	0.924***	0.991***	0.912***	0.851**	0.843**	0.970***	0.854**	0.978***	0.954***	0.969***	0.982***	0.960***
Ser					0.861**	0.858**	0.956***	0.830**	0.725*	0.708*	0.897***	0.916***	0.965***	0.926***	0.862**	0.911***	0.908***
Glu						0.998***	0.948***	0.974***	0.936***	0.940***	0.979***	0.815**	0.946***	0.861**	0.933***	0.945***	0.993***
Pro							0.947***	0.975***	0.950***	0.955***	0.982***	0.794*	0.942***	0.855**	0.941***	0.952***	0.992***
Gly								0.933***	0.865**	0.855**	0.981***	0.875**	0.990***	0.948***	0.966***	0.984***	0.978***
Ala									0.911***	0.920***	0.963***	0.808**	0.910***	0.860**	0.942***	0.932***	0.971***
Val										0.991***	0.931***	0.616	0.841**	0.743*	0.919***	0.915***	0.925***
ILE											0.933***	0.602	0.836**	0.741*	0.918***	0.907***	0.925***
Leu												0.820**	0.971***	0.908***	0.983***	0.987***	0.993***
Tyr													0.905***	0.883**	0.767*	0.787*	0.846**
Phe														0.950***	0.938***	0.960***	0.974***
His															0.910***	0.899***	0.906***
Lys																0.985***	0.959***
Arg																	0.972***

*,** and *** indicate significance at P≤0.05,0.01,0.001 respectively

Correlations between amino acids and mineral nutrient concentrations in spelt wheat genotypes are given in Table 3-19. In spelt wheat K was the only nutrient which had no correlation with any of the amino acids except Met sln (r=0.687, $P< 0.05$). Among nutrients, N and Mn have shown significant positive correlation with all of the amino acids. P had shown significant positive correlation with all of the amino acids except with Met sln. Ca was found to correlate negatively with all of the amino acids except Val, Ile, Lys and Arg. Among Mg, S, Ca, Fe and Cu, the least significant correlation with amino acids was found with Cu.

Table 3-19: Correlations between amino acids and nutrients in spelt wheat genotypes

	N	K	P	Mg	S	Ca	Fe	Mn	Cu	Zn
Cyst acid	0.955***	0.528	0.878**	0.788*	0.899***	-0.811**	0.854**	0.801**	0.709*	0.858**
Met sln	0.716**	0.687*	0.567	0.571	0.649	-0.430	0.531	0.791*	0.428	0.618
Asp	0.912***	0.523	0.813**	0.687*	0.704*	-0.821**	0.699*	0.946***	0.611	0.628
Thr	0.916***	0.510	0.753*	0.626	0.723*	-0.688*	0.691*	0.939***	0.533	0.701*
Ser	0.874**	0.480	0.695*	0.562	0.648	-0.759*	0.595	0.886***	0.457	0.620
Glu	0.981***	0.573	0.905***	0.820**	0.924***	-0.709*	0.891***	0.921***	0.757*	0.891***
Pro	0.976***	0.559	0.883**	0.798**	0.920**	-0.682*	0.883**	0.913***	0.731**	0.902***
Gly	0.943***	0.547	0.818**	0.696*	0.770*	-0.717*	0.745*	0.961***	0.615	0.728*
Ala	0.986***	0.532	0.906***	0.770*	0.903***	-0.695*	0.880*	0.875*	0.712*	0.865*
Val	0.890***	0.618	0.802**	0.738*	0.889***	-0.438	0.826**	0.849**	0.666*	0.879**
Ile	0.888***	0.539	0.802**	0.757*	0.902***	-0.479	0.864**	0.844**	0.697*	0.914***
Leu	0.957***	0.529	0.843**	0.740*	0.838**	-0.676*	0.818**	0.960***	0.674*	0.820**
Tyr	0.874**	0.491	0.766*	0.578	0.641	-0.854**	0.586	0.830**	0.502	0.550
Phe	0.936***	0.537	0.811**	0.708*	0.768*	-0.770*	0.739*	0.966***	0.625	0.729*
His	0.876**	0.566	0.778*	0.684*	0.686*	-0.805**	0.676*	0.908***	0.584	0.596
Lys	0.916***	0.522	0.797**	0.681*	0.774*	-0.600	0.763*	0.943***	0.613	0.749*
Arg	0.922***	0.513	0.781*	0.666*	0.771*	-0.605	0.749*	0.949***	0.593	0.763*
sum_w/o Trp	0.979***	0.570	0.875**	0.776*	0.878**	-0.717*	0.843**	0.944***	0.701*	0.845**

*,** and *** indicate significance at P≤0.05,0.01,0.001 respectively

When the relationships among macro and micro nutrients were evaluated in the spelt wheat genotypes, it has been found that K did not correlate with any of the other nutrients except P (r=0.67, $P<0.05$). In general grain Ca concentration tended to express negative correlations with the other nutrients. However, Cu and Zn. N, P, Mg, S, Fe, Mn and Cu have shown significant positive correlations among each other (Table 3-20).

Table 3-20: Correlations among nutrients in spelt wheat genotypes

	K	P	Mg	S	Ca	Fe	Mn	Cu	Zn
N	0.593	0.918***	0.778*	0.906***	-0.746*	0.856**	0.885**	0.707*	0.847**
K		0.670*	0.631	0.624	-0.299	0.475	0.520	0.505	0.379
P			0.923***	0.915***	-0.729*	0.921***	0.812**	0.894**	0.771*
Mg				0.891***	-0.692*	0.938***	0.733*	0.976***	0.759*
S					-0.630	0.953***	0.729*	0.832**	0.942***
Ca						-0.671*	-0.684*	-0.642	-0.554
Fe							0.743*	0.927***	0.906***
Mn								0.688*	0.682*
Cu									0.714*

*,** and *** indicate significance at P≤0.05,0.01,0.001 respectively

3.4.3 Analysis of wild wheat genotypes

Fourteen wild wheat genotypes (*T. dicoccoides*) were evaluated for the concentrations of Cyst Acid, Meth sln, Asp, Thr, Ser, Glu, Pro, Gly, Ala, Val, Ile, Leu, Tyr, Phe, His, Lys and Arg. Each amino acid and the sum of the amino acids have been investigated to find out the possible correlations with the mineral nutrients (i.e. N, K, P, Mg, S, Ca, Fe, Mn, Cu and Zn) analyzed in the whole meal flour of the genotypes.

As consistent with other wheat species, concentration of Glu was the highest among the amino acids ranging between 5.80 and 7.34 g/100g wf. In average, Glu constituted about 35% of the total amino acids in the wild wheat grains (Table 3-21). The second highest amino acid was Pro and ranged between 1.61 and 2.48 g/100 g wf. The share of Pro in total amino acid concentration was 11%. Met sln had the lowest amino acid concentration ranging between 0.255 and 0.434 g/100 g wf. Lys had the second lowest concentration ranging between 0.316 and 0.41 g/100 g wf.

When the variations among the genotypes were investigated, it was found that Cyst acid was highly stable among amino acids analyzed showing only a little variation (% cv: 6) (Table 3-21) which was consistent with modern and spelt wheat species. In addition to Cyst acid , Glu also had a very low variation among genotypes (% cv: 8). The highest variation was expressed with Arg with a cv of 26%, followed by Ile with a cv of 24 %.
The total amino acid content ranged between 14.9 g/100 g wf (TD 536) and 21.1 (TTD 86) with an average of 18.3 g/100 g wf. The coefficient of variation for the total amino acid content was calculated as 11 %.

3-21: Concentrations of amino acids in wild wheat genotypes

Genotype	Cyst acid	Met sln	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Val	Ile	Leu	Tyr	Phe	His	Lys	Arg	Total
									g/100 g wf									
TD 536	0.927	0.367	0.641	0.251	0.648	5.80	1.61	0.607	0.545	0.298	0.185	0.652	0.447	0.722	0.395	0.340	0.510	14.9
TD 531	0.869	0.363	0.783	0.395	0.876	6.98	2.04	0.665	0.607	0.616	0.475	1.142	0.627	1.046	0.416	0.379	0.546	18.8
TD 510	0.985	0.304	0.741	0.369	0.823	5.96	1.76	0.627	0.615	0.595	0.461	0.961	0.483	0.835	0.378	0.363	0.526	16.8
TTD 27	0.985	0.304	0.670	0.364	0.794	5.94	1.76	0.420	0.466	0.427	0.300	1.007	0.559	0.844	0.436	0.326	0.479	16.1
TD 195	0.870	0.255	0.862	0.475	0.887	6.08	2.18	0.696	0.832	0.598	0.532	1.312	0.728	0.967	0.419	0.392	0.736	18.8
TD 391	0.925	0.323	0.868	0.498	0.884	6.02	1.86	0.615	0.797	0.674	0.552	1.163	0.646	0.833	0.365	0.413	0.744	17.0
TTD 28	1.044	0.290	0.804	0.351	0.878	6.31	1.96	0.717	0.643	0.574	0.506	1.270	0.747	0.854	0.428	0.336	0.679	18.4
TD 390	0.989	0.300	0.829	0.470	0.901	6.37	2.09	0.667	0.696	0.615	0.565	1.349	0.695	0.970	0.423	0.407	0.903	19.2
TTD 89	0.903	0.287	0.892	0.487	1.003	6.86	2.32	0.790	0.904	0.641	0.574	1.415	0.841	1.051	0.457	0.376	1.099	20.9
TD 636	0.954	0.435	0.901	0.494	1.093	7.06	2.29	0.757	0.949	0.775	0.645	1.435	0.634	1.072	0.502	0.510	0.861	21.0
TTD 86	0.974	0.411	0.893	0.494	0.935	6.58	2.20	0.727	0.759	0.779	0.556	1.408	0.650	1.061	0.472	0.404	0.819	20.1
TTD 75	1.042	0.432	0.994	0.504	0.994	7.34	2.48	0.810	0.716	0.644	0.502	1.380	0.732	1.031	0.531	0.463	0.710	21.1
TD 399	1.004	0.434	0.670	0.293	0.548	5.80	1.89	0.631	0.861	0.712	0.536	1.119	0.372	0.682	0.364	0.472	0.662	17.0
TTD 18	0.876	0.266	0.388	0.305	0.739	6.25	1.78	0.514	0.675	0.674	0.557	0.772	0.386	0.544	0.269	0.316	0.523	15.8
mean	0.953	0.341	0.781	0.411	0.857	6.38	2.01	0.660	0.719	0.616	0.496	1.170	0.611	0.894	0.418	0.393	0.700	18.3
stdev	0.060	0.065	0.152	0.089	0.143	0.50	0.25	0.105	0.140	0.127	0.119	0.247	0.143	0.163	0.065	0.058	0.179	2.0
cv (%)	6	19	20	22	17	8	13	16	19	21	24	21	23	18	16	15	26	11

Among the macro nutrients analyzed in wild wheat genotypes, concentrations of Ca (range: 0.054-0.091%) showed the largest variation (Table 3-22). There was no significant variation in the N, K, P, Mg or S concentrations. In regard of the micronutrients, concentration of Mn (range: 37.9-77.2 mg kg^{-1}) and Cu (range: 3.07-8.40 mg kg^{-1}) were found to have the highest variation Compared to Mn and Cu, concentration of Fe (range: 38.9-59.9 mg kg^{-1}) and Zn (range: 69.6-40.6 mg kg^{-1}) had less variation among the wild wheat genotypes analyzed.

3-22 : Concentrations of nutrients in wild wheat genotypes

Genotype	N	K	P	Mg	S	Ca	Fe	Mn	Cu	Zn
			%					mg kg^{-1}		
TD 536	3.70	0.530	0.590	0.140	0.220	0.0709	36.4	46.7	3.93	44.5
TD 531	4.40	0.520	0.580	0.140	0.230	0.0528	42.3	68.6	5.10	40.6
TD 510	3.80	0.501	0.565	0.130	0.240	0.0552	38.9	77.2	6.36	49.0
TTD 27	4.00	0.552	0.570	0.150	0.256	0.0769	49.1	65.1	6.34	69.6
TD 195	3.92	0.651	0.643	0.156	0.256	0.0913	53.5	45.4	5.48	67.7
TD 391	3.84	0.540	0.620	0.149	0.264	0.0541	58.6	75.7	5.18	52.1
TTD 28	4.45	0.539	0.579	0.155	0.272	0.0613	46.9	74.4	5.86	67.8
TD 390	3.99	0.540	0.612	0.163	0.260	0.0619	59.9	76.3	5.60	56.0
TTD 89	4.38	0.545	0.567	0.144	0.238	0.0563	47.9	71.9	5.39	67.8
TD 636	4.44	0.574	0.582	0.142	0.251	0.055	44.2	44.2	3.89	49.0
TTD 86	4.32	0.498	0.526	0.141	0.269	0.0625	49.7	47.9	4.85	56.0
TTD 75	4.99	0.619	0.617	0.159	0.270	0.0736	46.2	40.2	8.40	54.0
TD 399	3.97	0.633	0.619	0.148	0.250	0.0723	45.2	68.7	3.07	52.8
TTD 18	3.73	0.568	0.500	0.125	0.234	0.0599	38.9	37.9	4.77	54.6
mean	4.14	0.558	0.584	0.146	0.251	0.065	47.0	60.0	5.30	55.8
stdev	0.37	0.047	0.038	0.011	0.016	0.011	7.0	15.2	1.29	9.2
cv (%)	9	8	7	7	6	17	15	25	24	16

Correlations among individual amino acids of wild wheat genotypes are provided in Table 3-23. There was no correlation found between either Cyst acid or Met sln with the remaining amino acids (except between Met sln and Lys). The third least correlating amino acid was Val which showed no correlation with 11 out of 16 amino acids. On the other hand, Leu, Gly and Thr showed the highest number of correlations; these had correlated with at least 12 out of 16 amino acids. There were also significant correlations among Asp, Thr, Ser, Glu, Pro, Gly, Leu, Tyr, Phe, His, Lys, and Arg, but with a few exceptions (i.e. Thr - Lys, Ser - Lys, Glu – Lys, Glu- Arg, Phe – Lys , His-Lys and His–Arg showed no correlation). The sum of all of the amino acids correlated well with all amino acids except Cyst acid and Met sln.

3-23: Correlations among amino acids in wild wheat genotypes

	Met_sln	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Val	Ile	Leu	Tyr	Phe	His	Lys	Arg	sum_w_o_Trp
Cyst_acid	0.387	0.287	0.002	0.010	0.030	0.115	0.174	-0.134	0.028	-0.038	0.249	0.066	0.057	0.411	0.259	0.013	0.154
Met_sln		0.326	0.100	0.042	0.375	0.316	0.363	0.261	0.321	0.064	0.229	-0.166	0.258	0.480	0.740**	0.053	0.324
Asp			0.831***	0.737**	0.571*	0.773***	0.779***	0.445	0.325	0.357	0.844***	0.804***	0.877***	0.837***	0.586*	0.640*	0.806***
Thr				0.864***	0.615*	0.806***	0.602*	0.544*	0.553*	0.614*	0.847***	0.773***	0.807***	0.625*	0.510	0.717**	0.808***
Ser					0.779***	0.779***	0.629*	0.393	0.427	0.528	0.759**	0.803**	0.829***	0.692**	0.339	0.624*	0.828***
Glu						0.839***	0.692**	0.334	0.457	0.465	0.634*	0.584*	0.724**	0.669**	0.438	0.464	0.847***
Pro							0.827***	0.618*	0.576*	0.624*	0.886***	0.739**	0.797***	0.755**	0.608**	0.723**	0.970***
Gly								0.629*	0.461	0.517	0.742**	0.663**	0.692**	0.657*	0.573*	0.703**	0.854***
Ala									0.738**	0.788***	0.637*	0.316	0.331	0.221	0.714**	0.759**	0.610*
Val										0.929***	0.632*	0.178	0.316	0.127	0.616*	0.508	0.602*
Ile											0.699**	0.361	0.342	0.105	0.538*	0.649*	0.652*
Leu												0.795***	0.821***	0.720**	0.606*	0.796***	0.925***
Tyr													0.798***	0.674**	0.151	0.702**	0.764***
Phe														0.853****	0.442	0.605*	0.865***
His															0.504	0.471	0.780***
Lys																0.460	0.600*
Arg																	0.770***

*,** and *** indicate significance at P≤0.05,0.01,0.001 respectively

Correlations between the amino acids and mineral nutrient concentrations in wild wheat genotypes are provided in Table 3-24. Among the nutrients, K, P, Mg, Ca, Mn, Cu and Zn showed no correlation with any of the 16 amino acids analyzed. However, concentration of N correlated positively well with all amino acids except Met sln, Thr, Ala, Val, Ile, Lys and Arg. There were also significant correlations with S and Cyst acid, Asp, Thr and Leu and with Fe and Thr, Leu, Tyr and Arg. Among the mineral nutrients, Ca in particular tended to have negative relationships with most of the amino acids.

3-24: Correlations between amino acids and nutrients in wild wheat genotypes

	N	K	P	Mg	S	Ca	Fe	Mn	Cu	Zn
Cyst. acid	0.401	-0.004	-0.131	0.433	0.611*	0.062	0.101	0.213	0.361	0.165
Met. sln	0.472	0.099	0.221	-0.024	0.114	-0.081	-0.202	-0.288	-0.208	-0.526
Asp	0.675**	0.068	0.046	0.479	0.595*	-0.011	0.506	0.116	0.383	0.120
Thr	0.520	0.072	-0.127	0.308	0.574*	-0.105	0.654*	-0.022	0.390	0.198
Ser	0.646*	-0.108	-0.087	0.134	0.402	-0.287	0.342	-0.096	0.434	0.173
Glu	0.871***	0.033	-0.010	0.047	0.170	-0.310	0.003	-0.273	0.364	-0.122
Pro	0.816***	0.337	-0.020	0.348	0.483	0.032	0.333	-0.255	0.345	0.198
Gly	0.711**	0.156	0.058	0.246	0.291	-0.142	0.130	-0.103	0.168	-0.031
Ala	0.243	0.434	0.114	0.071	0.199	-0.097	0.298	-0.119	-0.324	0.079
Val	0.354	0.148	-0.030	-0.077	0.364	-0.354	0.265	-0.068	-0.113	-0.064
Ile	0.328	0.219	-0.174	0.068	0.365	-0.338	0.397	0.030	-0.048	0.101
Leu	0.696**	0.196	-0.130	0.473	0.687**	-0.046	0.597*	0.092	0.242	0.333
Tyr	0.626*	0.021	-0.093	0.508	0.522	-0.023	0.559*	0.158	0.482	0.473
Phe	0.692**	-0.099	-0.075	0.253	0.397	-0.109	0.367	0.037	0.323	0.076
His	0.805***	0.066	0.059	0.391	0.505	0.117	0.209	-0.145	0.394	0.154
Lys	0.451	0.421	0.055	0.240	0.327	-0.023	0.240	-0.151	-0.147	-0.281
Arg	0.385	0.091	-0.283	0.236	0.337	-0.192	0.540*	0.125	-0.031	0.313
sum. w. o Trp	0.815***	0.155	-0.093	0.276	0.462	-0.135	0.325	-0.111	0.281	0.152

*,** and *** indicate significance at P≤0.05,0.01,0.001 respectively

The relationships among nutrients in the wild wheat genotypes are shown in Table 3-25. Among macro and micro nutrients positive correlations were found between Ca-K (r=0.692, *P*<0.01), Mg-S (r=0.71, *P*< 0.01), Mg-Ca (r=0.536, *P*< 0.05), Mg-Fe (r=0.665, *P*< 0.01), Fe-S (r=0.681, *P*<0.01), and S-Zn (r=0.533, *P*< 0.05). No positive correlation was found among other nutrients.

3-25: Correlations among nutrients in wild wheat genotypes

	K	*P*	*Mg*	*S*	*Ca*	*Fe*	*Mn*	*Cu*	*Zn*
N	*0.146*	*0.151*	*0.337*	*0.459*	*-0.089*	*0.055*	*-0.155*	*0.476*	*0.090*
K		*0.259*	*0.506*	*0.225*	*0.692***	*0.146*	*-0.397*	*0.016*	*0.287*
P			*-0.171*	*-0.083*	*0.190*	*-0.415*	*-0.261*	*-0.038*	*0.008*
Mg				*0.710***	*0.536**	*0.665***	*0.126*	*0.352*	*0.460*
S					*0.275*	*0.681***	*0.079*	*0.452*	*0.533**
Ca						*0.138*	*-0.394*	*0.140*	*0.447*
Fe							*0.341*	*0.183*	*0.429*
Mn								*0.034*	*0.111*
Cu									*0.308*

*,** and *** indicate significance at *P*≤0.05, 0.01, 0.001 respectively

4 Discussion

Nutrition is the main requirement of all living organisms. The socioeconomic well-being of populations is directly related to the ease of access to adequate and nutritious food sources. During the past few decades, adoption of the green revolution cropping systems provided many nations with inexpensive and sufficient food. However, this has brought about the classical quantity or quality problem, leading to endless debates where increasing number of millions of malnourished people are on one side and others looking for enhanced low-calorie diets on the other. Having information about (Basak, et al., 1993; Hirs, et al., 1954) and increasing the nutritious value of staple food crops in a sustainable way (Welch and Graham, 1999; Cakmak et al, 2002) is a challenging target in which both developed and developing nations can benefit. As discussed before, wheat constitutes a major portion of daily calorie intake particularly in the developing nations. The nutritious value of wheat is usually determined by the protein content. However, the amino acid profile itself rather than the bulk protein content is a distinct quality parameter together with grain mineral nutrient content. Currently grain protein is determined by instrumental analysis techniques mainly involving analysis of N. Once grain N is determined it is converted to protein by calculation using conversion factors and determination of individual amino acids is routinely applied for large number of samples (e.g. for screening purposes) mainly because of the need for investment of expensive instrumentation and lack of a rapid and practical method for hydrolysis of cereal grains. Therefore, the main focus of this thesis study was the development of an expedient hydrolysis method for the wheat grain, the major source of protein and calorie intake in the developing world. Following method development, modern, primitive and wild wheats were subjected to the newly developed rapid microwave-assisted hydrolysis and thereafter the amino acid profiles of wheat species and genotypes were determined to assess the relationships between individual amino acids and mineral nutrients in wheat.

4.1. Utilization of microwave-assisted protein hydrolysis for wheat

The main focus of this study was to use microwave radiation to accelerate the processing and to improve the analysis quality of wheat protein hydrolysis. Microwaves are one sub-class of electromagnetic radiation, which have been used as a heating method. Unlike conventional heating methods, microwaves have the ability to penetrate samples and to heat uniformly and rapidly. Specific techniques related to laboratory microwaves have evolved over the past two

decades to include models specific to protein hydrolysis (break the peptide bonds under acidic conditions) (Lill, et al., 2007). Other developments about microwaves have been discussed in section **Error! Reference source not found.**. To the best of our knowledge, no microwave assisted hydrolysis method has been discussed in the literature that specifically describes the hydrolysis of wheat flour proteins. Accordingly, this study was focused on such proteins. In this study, the optimum conditions for the microwave-assisted hydrolysis of wheat flour protein were assessed using Balcali 2000 as model wheat flour. Parameters, which were specifically optimized, were the sample mass, hydrolysis time and temperature.

As with the other acid-promoted hydrolysis methods, several unavoidable side-reactions were experienced in the use of microwave heating. Tryptophan, for instance, was completely destroyed. Glutamine and asparagine were converted to glutamic acid and aspartic acid; hence, the combined acid and amide forms were quantified with no possibility of achieving individual resolution. Cystine, cysteine and methionine were partially degraded by hydrolysis but they could be measured quantitatively in subsequent trials after pre-oxidizing the protein samples using performic acid at 0^{o}C. Under such conditions, cysteine and cystine were smoothly converted to cysteic acid (Cyst acid), whereas methionine became methionine sulfone (Met sln). The chemistry of these sulfur amino acids will be discussed in a following section. All other amino acids were quantitatively measured by this method and the overall results were compared against traditional acid-based hydrolysis techniques such as the reflux method.

Ninhydrin is known to react with the amino groups of all amino acids. That being said, the chemistry of ninhydrin differs between primary and secondary amino groups in the sense that the amino group of primary amines is cleaved to yield two molecules, namely, a chromophore and a carboxyaldehyde; in comparison, the pyrolidine-type amino group of proline forms an addition product with ninhydrin, yielding a single molecule as product. Moreover, primary amino groups yield a highly colored chromophore, namely, Ruhemann's Purple (λ_{max} = 570 nm) during reaction with ninhydrin, whereas proline, the only amino acid in proteins to bear a secondary amino group, produces a brightly colored yellow chromophore. More specifically, ninhydrin and proline form a 1:1 imino-bridged complex (λ_{max} = 440 nm) over the course of this reaction (Laskar, et al., 2001; Friedman, 2004). Another interesting point is the fact that lysine, with twice the number of amino groups

compared to the other amino acids, displays an approximate ninhydrin color yield of only 1.2 with respect to glycine. This discrepancy has been rationalized by the formation of a cyclic imine complex between the amino and aldehyde groups of the aminocarboxyaldehyde intermediate. Despite these interesting differences of chemistry, the chromatographic detection of ninhydrin-processed amino acids has been adequately optimized by fine-tuning of the reaction conditions as well as the use of carefully calibrated standards. Indeed, ninhydrin analyses using this intrinsically-sensitive chromophoric method for detection have easily permitted the sub-micromole detection of amino acids. In the work presented herein, ninhydrin was used as the detection reagent in a Biochrom 30 model amino acid analyzer (HPLC).

This quantification method, like many other quantification methods, was consistent in its own right. Still, the nature of the wheat samples, just as in the case of other plant-based analytes, had introduced complexities that would not normally have been an issue in classic samples such as soluble and purified proteins. In particular, the recoveries expressed for wheat sample were prone to greater error, and therefore the apparent values indicated were typically used to make relative comparisons. For instance, acid hydrolysis was not, at least initially, a homogenous phase process. In order to hydrolysis to proceed, aqueous acid must have swollen and penetrated the wheat flour matrix; the acid solution must have found the target sites and achieved hydrolysis; and lastly, the hydrolyzed products must have diffused away in order to allow further penetration of acid. Expressed in more definite terms, the chemistry of hydrolysis was necessarily influenced by at least three factors. One limiting factor was conceivably the bulk-phase permeation of solution into the reaction sites; such an event would be necessary to establish a local hydrolysis zone, which would follow heterogeneous-phase kinetics. Similarly, the same argument would apply for products yielded in this local hydrolysis zone; in order for any hydrolysis to continue, products presumably would need to escape the confines of the wheat matrix, which is again governed by bulk-phase diffusion. A third factor to consider would be the individual species diffusion of acid and water towards the amide bonds, as well as reverse diffusion of molecular products. The weight average of these three factors would define the apparent yield of accessible amino acids. Further compounding this issue was the color yield of ninhydrin. While ninhydrin is known to react effectively with amino acids, the hydrolysis environment of wheat would be expected to contain a great number of potential interferents in comparison to the hydrolysate of a typical purified protein. In particular, the *in situ* creation of aldehydes, arising from the destruction of

carbohydrates, lipids and nucleic acids, would be expected to compete with ninhydrin for the accessible amino groups, potentially causing an attenuated color yield. Hence, the "protein recovery" in the strictest sense can only be an apparent recovery, which is a weighted average of the accessible amino acid content of a sample (destroyed amino acids notwithstanding) and the ninhydrin color yield in the presence of interferents.

Total wheat flour from a model durum wheat (*T. durum*) Balcali-2000 was subjected to microwave-assisted hydrolysis in 5ml 6 N hydrochloric acid. Samples weighing 100, 200, 300, 400 and 500 mg were incubated at 130, 150 or 170^{o}C for 1, 2, 3, 4 or 5h time intervals. The hydrolysates were passed through a 0.2um nitrocellulose filter and subjected thereafter to automated amino acid analysis. All amino acids were quantified with exception to methionine, cysteine and tryptophan, which were destroyed during hydrolysis. Asparagine and glutamine were quantified as converted to their respective carboxylic acids (i.e. aspartic acid: Asp and glutamic acid: Glu). Overall, the greatest ninhydrin color-yields were obtained after hydrolyzing 200 mg samples at 150^{o}C for 3h. Samples exceeding 200 mg typically afforded increasingly lower ninhydrin color-yields. An exception was tyrosine, which displayed a notably maximum color yield at 100 mg of sample. The optimal hydrolysis temperature of 150^{o}C appeared to strike a balance between liberating amino acids from the wheat matrix as well as limiting their premature destruction. Likewise, the optimum reaction time of 3h was observed to strike a balance between maximizing hydrolysis and minimizing sample loss, the latter aspect contributing more strongly at longer reaction times. Furthermore, a comparison against the total nitrogen content of these samples indicated that the overall ninhydrin color-yield was 85% (without Triptophan, Cysteine and Methionine) when compared with total protein result as calculated over total N. The highest amino acid content pointed to the combined contributions of glutamic acid and glutamine, in accord with previous findings. Also as expected, proline was found to rank in second place. It follows to reason that an optimized microwave-assisted hydrolysis method may describe a rapid means to compare the constitution of different genotypes of wheats and may further show merit and general applicability towards the rapid analysis of important cereals of commercial interest.

Leu, Ile and Val are the most hydrophobic amino acids and for this reason, their juxtapositioning along the primary chain results in an exceptionally-resistant, hydrolytically stable bond (Roach, et al., 1970). This characteristic may have explained the finding that Leu was recovered more quantitatively, in relative terms, following treatment at 170^{0}C as opposed

to 150°C or 130°C. While the reason for this finding was not conclusively determined, it would appear that the other amino acids were cleaved earlier in the hydrolysis reaction in view of their higher accessibility to acid: presumably, these residues, once freed, were subjected to the destructive effects of the environment for a longer period. Also, Ile was recovered more after treatment at 170 ^{0}C, followed by 150 ^{0}C and then 130 ^{0}C. Although the Val results were not statistically different at 150 and 170°C, the apparent recovery of Val hydrolyzed at 170 ^{0}C was clearly the highest (0.59 g/ 100 g wf), followed by 150 ^{0}C (0.52 g/ 100 g wf) and finally 130 ^{0}C (0.34 g/ 100g wf) which had produced significantly lower values.

Tyr recovery at 130^{0}C (0.37 g/100 g w f) was higher than at 150 ^{0}C (0.33 g/100 g w f) or at 170 ^{0}C (0.28 g/100 g w f). Again, while the underlying reason to explain this trend was not proven directly, the destruction of tyrosine by chlorination of the ring is a well-documented and thermally-promoted fact. For this reason, the considerably reduced, apparent recovery of tyrosine at 170°C or even at 150°C was not surprising.

Looking to the effects of hydrolysis time, the apparent recovered quantities of all of the amino acids were significantly higher at 3h, revealing that 3h of hydrolysis at the given conditions was the optimum compared to 1, 2 or 4 h of microwave-assisted hydrolysis.

Looking to the effect of sample mass, recovery of all amino acids (with exception to His and Tyr - which both had higher recoveries at 100 mg) were significantly higher when 200 mg of wheat flour was hydrolyzed at 150 ^{0}C for 3 h.

The optimal hydrolysis temperature of 150°C appeared to strike a balance between liberating amino acids from the wheat matrix as well as limiting their premature destruction. Likewise, the optimum reaction time of 3h when using 200 mg mass of sample was observed to strike a balance between maximizing hydrolysis and minimizing sample loss.

The theoretical total protein content of wheat flour is calculated by multiplying the %N with 5.83 as mentioned in section 1.2. Balcali 2000 durum wheat %N is 2.207, therefore; the theoretical total protein is 12.87 g. The recoverable percentage is 85.5% for the sum of the amino acids that were assisted in the non-oxidized method leaving 14.5% for Trp, Cyst acid,

Meth sln, non proteinous nitrogenous compounds and the loss of amino acids during acid hydrolysis.

Cysteine and methionine were substantially destroyed in the course of employing the classic hydrolysis conditions; hence, these amino acids were measured using a parallel method, which employed a pre-oxidation step using performic acid. Following performic acid oxidation and hydrolysis, cysteine and/or cystine was identified as cysteic acid whereas methionine could be identified as methionine sulfone.

The highest recovery (or more precisely, the highest ninhydrin color yields) of samples containing both Cyst acid and Met sln was obtained by applying a 16 h performic acid oxidation to 50 mg of wheat sample at 0^{o}C. Upon completion of oxidation, the performic acid was quenched as per the European Union protocol (EU Directive 98/64/EC, 1998) and the oxidized sample was hydrolyzed without delay by the addition of 10 ml 6 N HCl (3 h, 150^{o}C).

Currently, the reflux method is the most common protein hydrolysis method, which has been adopted by the European Union and USA (EU Comisson directive 98/64/EC, 1998, AOAC Official Method 994.12, 1995). For the most part, it features a high percentage recovery and reliability. However, the reflux method is time consuming to the extent that it inadvertently detracts from the productivity of such analyses and in certain cases it may in fact determine the rate of more comprehensive work. By referring to Table 3.5 and Figure 3.1, it is clear that the Sabanci University laboratory hydrolysis method featured comparable, if not better, results as those that were obtained by the traditional reflux method. More importantly, the Sabanci University laboratory analyses were achieved in a fraction of the time, as the microwave-assisted hydrolysis method is much more rapid.

4.2. **Relationship of amino acids with mineral nutrients in the wheat grain**

Wheat is one of the oldest diets of mankind. It contains carbohydrates, proteins, vitamins and minerals (as Mg, Mn, Cu, P, Fe, Zn and others) (Chess, et al., 2001). There are four types of wheat proteins and they differ in their solubility: Albumins which are soluble in water, globulins which are soluble in salt solution but not in water, gliadins which are soluble in 70-

90% ethanol and glutenins which are soluble in base but not in alcohol, saline solutions or neutral aqueous solutions (Osborne, 1907; Spurway, 2008).

Amino acid composition is a key factor in determining the wheat grain nutritional quality. Wheat protein is usually low in essential amino acids that are important part of human diet, particularly Lys (the most deficient amino acid) and Thr (ranking second after Lys), in the other hand they are rich in Glu (the most abundant amino acid in wheat grain) and Pro (the second highest one). Protein and amino acid composition of wheat highly fluctuates with both genotype and environmental characteristics such as N application time, fertilization rate, residual soil N and temperature during grain-filling (Luis, et al., 2007). In all wheat species and genotypes tested throughout this study, grain amino acid concentrations were found highest in Glu followed by Pro. Concentration of Glu ,whereas Met sln, Lys, Thr and were the lowest amino acids (Tables 3-6, 3-7, 3-16 and 3-21). The quantities and ratios of individual amino acids detected by the hydrolysis method used in this study were consistent with the literature data (Boila et al., 1996) and the quantitative order of major and minor amino acids in wheat do not change considerably. As an example, the grain concentration of Cyst acid had an exceptionally high stability among all genotypes and species (see Tables 3-6, 3-7, 3-16 and 3-21). Therefore, it seems there is little scope for selection or breeding for high cysteine content in wheat. However, other minor or major amino acids exhibited significantly high variations among genotypes and species.

In wild wheats, the concentration of major (e.g. Glu and Pro) and minor amino acids (e.g. Met sln, Lys, His) were 1.5-2 fold higher than modern bread or durum wheat species (Tables 3-6, 3-7 and 3-21). Accordingly, the average of total amino acids present in wild wheats was 83% higher than durum and 71% higher than bread wheats. In previous studies wild emmer wheat (*T. dicoccoides*) was proposed to be an important genetic resource for increasing micronutrients in modern cultivated wheat (Cakmak et al., 2000, 2004; Peleg et al., 2008) because of the exploitable genetic variation in their grain nutrient concentration. Results presented in this thesis study show that the higher amino acid content of wild emmer wheats also constitutes as a valuable genetic resource for increasing individual or total amino acids (i.e. proteins) in modern wheats.

The total amino acid concentration of selected spelt wheat genotypes fell in between modern and wild wheats. However, in spelt wheat genotypes the variations in individual or total

amino acids were substantially higher compared to modern or wild wheats mainly due to prescreening for high and low grain N (Table 3-16). Nevertheless, spelt wheat can also be considered as a breeding material for increasing the nutritional quality of cultivated wheats. The range of N concentration in spelt genotypes (i.e. four low-N and five high-N genotypes) provided by prescreening had affected not only the variation in amino acids, but also mineral nutrient concentrations (Tables 3-16 and 3-17). The contrasting N values were exposed with significant differences in virtually all nutrients (except Ca) as well as amino acids (Tables 3-16 and 3-17). Moreover, varied grain N resulted in highly significant positive correlations among amino acid, amino acid-mineral nutrient and mineral nutrient concentrations of spelt genotypes (Tables 3-18, 3-19 and 3-20). Similarly, amino acids in modern and wild wheats also correlated well within and among each other. However, amino acids in both modern and wild wheats had noticeably fewer correlations with mineral nutrients (Tables 3-11, 3-14 and 3-24).

Among mineral nutrients only N and S and partly Zn and Fe had correlated well with only a few of the amino acids. In the case of spelt wheat, mineral nutrients (except K, Ca and Cu) had correlated well with a majority of amino acids including the sum of amino acids. Interestingly Ca had a significant but negative correlation with a majority of amino acids and mineral nutrients in spelt wheat (Table 3-24). Although not significantly expressed, this negative association of Ca with amino acids was also evident in modern and wild wheats. Currently there is no published data available on the relationship of grain Ca with protein or amino acids. Considering the well-documented poor phloem mobility of Ca (Marschner, 1995), the negative correlations could hardly be associated with an inhibitory effect of Ca on transport of amino acids into wheat grain. On the contrary, the negative correlation of Ca with grain mineral nutrients and amino acids could be a consequence of an enhanced phloem loading (i.e. with amino acids and other mineral nutrients during senescence) which disturb phloem mobility and deposition of Ca into grains.

This study characterizes the amino acid profiles of a number of cultivated and wild wheat genotypes. It is proposed that a substantial genetic variation is present in spelt and wild emmer wheats which can be exploited to enhance specific and/or total amino acids (i.e. protein) in high yielding cultivated wheats through selection, breeding and targeted molecular approaches.

Although the existence of significant associations between a few amino acids and mineral nutrients, these associations were not found consistent among species and genotypes. Thus, it was not possible to define or explain a co-transport or co-accumulation mechanism of specific amino acids with mineral nutrients. In light of this evidence, future research should focus on the phloem transport and mobility of metal binding proteins and organic ligands, rather than individual amino acids.

A major finding of this study was the augmentation of correlations (among amino acids, nutrients and amino acids with nutrients) upon prescreening for contrasting grain N (or protein) concentration. It is concluded that advancements in increasing the grain protein content of wheat can significantly contribute to enrichment of grains with almost all mineral nutrients except K and Ca.

REFERENCES

Abderhalden, E., & Schmidt, H. (1911). On the use of triketohydrinden-hydrate to demonstrate proteins and their degradation products. *Journal Of Research In Physical Chemistry & Chemical Physics , 72*, 37-43.

Abedrhalden, E., & Schmidt, H. (1913). Some observations and experiments with triketohydrindenhydrate. *Journal Of Research In Physical Chemistry & Chemical Physics , 85*, 143-147.

Acquistucci, R., Degidio, M., & Vallega, V. (1995). Amino acid composition of selected strains of diploid wheat, Triticum monococcum L. *Cereal Chemistry , 72*, 213-216.

Albin, D. M., Wubben, J. E., & Gabert, V. M. (2000). The influence of hydrochloric acid concentration and measurment method on the determination of amino acid levels in soya bean products. *Animal Feed Science and Technology , 87*, 173-186.

Al-Shabanah, O., Raza, M., Al-Harbi, M., Al-Bekairi, A., Al-Ghararably, N., & Qureshi, S. (2000). Effect of ninhydrinon the biochemical and histopathological changes induced by ethanol in gastric mucosa of rats. *life Science , 67*, 559-566.

AOAC. (1995). AOAC Official Method 994.12 Amino Acids in Feed, Alternatives I and III *Official Methods of Analysis of AOAC International, Volume I, 16th Edition*, Arlington, Virginia, USA.

Azzeh, F., & A.S, A. (2009). Evaluation of protein content, lysine and sulfur-containing amino acids contentand electrophoretic patterns of soluble proteins for gamma-irradiated semolinabefore and after milling of durum wheat. *Radiation Physics and Chemistry , 78*, 971-975.

Barneix, A. (2007). Physiology and biochemistry of source-regulated protein accumulation in the wheat grain. J. *Journal of Plant Physiology , 164*, 581-590.

Basak, B., Bhattacharyya, U., & Laskar, S. (1993). Spray reagent for the detection of amino acids on thin layer chromatography plates. *Amino Acids , 4*, 193-196.

Bazinet, r. P., Rao, J. S., Chang, L., Rapoprt, S. I., & Lee, H.-J. (2006). Chronic Carbamazepine decreases the incorporation rate and turnover of Arachidonic Acid but not Docosahexaenoic Acid in brain phospholipids of the unanesthetized rat: relevance to bipolar disorder. *Biological Psychiatry , 59*, 401-407.

Beck, F., Kaplan, J., Fine, N., Handschu, W., & Prasad, A. (1997). Decreased expression of CD73 (ecto-5′-nucleotidase) in the CD8+ subset is associated with zinc deficiency in human patients1. *Journal of Laboratory and Clinical Medicine , 130*, 147-156.

Bernal-Lugo, I., Rodriguez, M., Gavilanes-Ruiz, M., & Hamabata, a. (1999). Reduced Aleurone α- amylase Production in Aged Wheat Seed is Accomplished by Lower Levels of

High-PI α- amylase Transcripts andReduced Response to Gibberellic Acid. *Journal of Experimental Botany , 50*, 311-317.

Bernstein, F., Koezle, T., Williams, G., Meyer, E., Brice, M., Rodgers, J., et al. (1977). The Protein Data Bank: a computer-based archival file for macromolecular structures. *Journal of Molecular Biology , 112*, 535-542.

Black, M. (2003). Micronutrient defeceincies and cognitive functioning. *Journal of Nutrition , 133*, 3927S-3931S.

Bohak, Z. (1964). N-epsilon-(dl-2-amino-2-carboxyethyl)-l-lysine, a new amino acid formed on alkaline treatment of proteins. *Journal of Biological Chemistry , 239*, 2878-2887.

Bohlen, P., & Schroeder, R. (1982). High-sensitivity amino acid analysis: Methodology for the determination of amino acid compositions with less than 100 picomoles of peptides. *Analytical Biochemistry , 126*, 144-156.

Boila, R.J., Stothers, S.C., Campbell, L.D. (1996). The relationship between the concentrations of individual amino acids and protein in wheat and barley grown at selected locations throughout Manitoba. *Canadian Journal of Animal Science* 76, 163–169.

Bouis, H. (2003). Micronutrient fortification of plants through plant breeding: can it improve nutrition in man at low cost? *Proceedings of Nutrition Society , 62*, 403-411.

Bouis, H., Graham, R., & Welch, R. (2000). The consultative group on international agricultural research (CGIAR) micronutrients project:justification and objectives. *Food and Nutrition Bulletin , 21*, 374-381.

Briddon, A., & Oberholzer, V. (1987). An alternative way of presenting quantitative amino acid results. *Clinical Chimica Acta , 164*, 209-213.

Buttrose, M. (1963). Ultrastructure of the developing wheat endosperm. *Australian Journal of Biological Sciences , 16*, 305.

Cakmak, I. (2008). . Enrichment of cereal grains with zinc: agronomic or genetic biofortification? *Plant and Soil , 302*, 1-17.

Cakmak, I., Graham, R., & Welch, R. (2002). Agricultural and molecular genetic approaches to improving nutrition and preventing micronutrient malnutrition globally. *Encyclopedia of Life Support Systems. UNESCO Publishing , 2*, 1075-1099.

Cakmak, I., Ozkan, H., Braun, H., Welch, R., & Romheld, V. (2000). Zinc and iron concentrations in seeds of wild, primitive and modern wheats. *Food and Nutrition Bulletin , 21*, 401-403.

Cakmak, I., Pfeiffer, W., McClafferty, B. (2010). Biofortification of Durum Wheat with Zinc and Iron. *Cereal Chemistry* , 87, 10-20.

Cakmak, I., Tolay, I., Ozdemir, A., Ozkan, H., Ozturk, L., & Kling, C. (1999). Differencies in zinc efficiency among and within diploid, tetraploid and hexaploid wheats. *Annals of Botany , 84*, 163-171.

Cakmak, I., Torun, E., Millet, E., Feldman, M., Fahima, T., Korol, A., et al. (2004). Triticum dicoccoides: an important genetic resource for increasing zinc and iron concentration in modern cultivated wheat. *Soil Science and Plant Nutrition* (50(7)), 1047-1054.

Caputo, C., & Barniex, A. J. (1999). The realtionship between sugar and amino acid export to the phloem in young wheat plants. *Annals of Botany , 84*, 33-88.

Castel, A., Kraal, B., Graaf, M., & Bosch, L. (1979). The primary structure of the coat protein of alfalfa mosaic virus strain VRU. *European Journal of Biochemistry , 102*, 125-138.

Chemguide. (2007). *http://www.chemguide.co.uk/analysis/chromatography/paper.html*.

Chen, SH, C., & T, W. K. (1991). Enhancement of chemical reactionsbby microwave irradiation. *Journal of the Chinese Chemical Society , 38*, 85-91.

Chess, A., Palmquist, D., & Rinke, J. (2001). Ionic ratios and crop performance: II. Effects of interactions amongst vanadium,phosphorus, magnesium and Ccalcium on soybean yield. *Journal of Agronomy and Crop Science , 187*, 47-52.

Clark, J. (2007). *http://www.youtube.com/watch?v=kDIiSsBxcwc&NR=1*.

Coleman, J. (1992). Zinc proteins: enzymes, storage proteins, transcription factors, and replication proteins. Annu. *Annual Review of Biochemistry , 61*, 897-946.

Condon, G. (1986). *Amino Acid analysis Theory and laboratory techniqes.* Cambridge, England.

Davis, M. (1986). *The Biochrom Handbook of Amino Acids.* Cambridge, England.

Demment, M. w., Youngy, M. M., & Sensenig, R. L. (2003). Providing micronutrients through food-based solutions: A key to human and national development. *The Journal of Nutrition , 133*, 3879S–3885S.

Distefeld, A., Cakmak, I., Peleg, Z., Ozturk, L., Yazici, A., Budak, H., et al. (2007). Multiple QTL-effects of wheat Gpc-B1 locus on grain protein and micronutrient concentrations. *Physiologia Plantarum , 129*, 635-643.

Dudev, T., & Lim, C. (2003). Principles governing Mg, Ca, and Zn binding and selectivity in proteins. *Chemical Reviews , 103*, 773-787.

Dupont, Chan, R., Lopez, W., & Vensel. (2005). Sequential extraction and quantitative recovery of gliadins, glutamins and other proteins from small samples of wheat flour. *Journal of Agricultural and Food Chemistry* (53), 1575-1584.

EC Directive. (1998). EC Commision Directive 98/64/EC of September Community methods for the determination of amino acids in feedstuff and amending Directive 71/393/ECC, annex part A, Determination of amino Acids. *Official Journal of the European Communities, 1998*.

Elango, R., Ball, R. O., & Pencharz, P. B. (2009). Amino acid requirements in humans: with a special emphasis on the metabolic availability of amino acids. *Amno Acids , 37*, 19-27.

FAO. (2009). *Share of dietry components in total energy consumption (2003-2005).*

Feller, U., & Fischer, A. (1994). Nitrogen metabolism in senescing leaves. Crit. Rev. *Plant Science , 13*, 241–273.

Ferney, H., Becerra, G., Yazici, A., Ozturk, L., & Cakmak, I. (2010). Genetic variation and environmental stability of grain mineral nutrient concentrations in Triticum dicoccoides under five environments. *Euphytica*, 171, 39–52.

Field, J., Shewry, P., & Miflin, B. (1983). Solubilization and characterization of wheat gluten proteins; correlations between the amount of aggregated proteins and baking quality. *Journal of the Science of Food and Agriculture , 34*, 370-377.

Fisher, D. B., & Macnicol, P. (1986). Amino acid composition along the transport pathway during. *Plant Physiology , 82*, 1019-1023.

Fountoulakis, M., & Lahm, H.-W. (1998). Hydrolysis and amino acid composition analysis of proteins. *Journal of Chromatography , 826*, 109-134.

Friedman, M. (2004). Applications of the ninhydrin reaction for analysis of amino acids, peptides, and proteins to agricultural and biomedical sciences. *Journal of Agriculture and Food Chemistry , 52*, 385-406.

Frossard, E., Bucher, M., Machler, F., Mozaffar, A., & Hurrel, R. (2000). Potential for increasing the content and bioavailability of Fe,Zn, and Ca in plants for human nutrition. *Journal of Agricultural and Food Chemistry* (80), 861-879.

Fujihara, S., Sasaki, H., Aoyagi, Y., & Sugahara, T. (2008). Nitrogen-to protein conversion factors for some cereal products in Japan. *Journal of Food Science , 73*, C204-C209.

Garcia Del Moral, L. F., Rharrabti, Y., Martos, V., & Royo, C. (2007). Environmentally induced changes in amino acid composition in the grain of durum wheat grown under different water and temperature regimes in a mediterranean environment. *Journal of Agriculture and Food Chemistry , 55*, 8144-8151.

Gepstein, S. (2004). Leaf senescence: not just a wear and tear phenomenon. *Genome Biology, 5*, 212.1–212.3.

Gibbon, B., & Larkins, B. (2005). Molecular genetic approaches to developing quality protein maize. *Trends in Genetics , 21*, 227-233.

Harry, E. (1951). The Effect of amino-acids on the growth of Lactobacillus casei A.T.C. 7469 in pteroyl glutamic acid-free med. *Biochemical Journal , 49*, 5-10.

Haydon, M., & Cobbett, C. (2007). Transporters of ligands for essential metal ions in plants. *New Phytologist , 174*, 499-506.

Hirs, C., Stein, W., & Moore, S. (1954). The amino acid composition of ribonuclease. *The Journal of Biological Chemistry , 211*, 941–950.

Hodisan, T., Culea B, m., Cimpoiu, C., & Cot c, A. (1998). Separation, identification and quantitative determination of free amino acids from plant extracts. *Journal of Pharmaceutical and Biomedical Analysis , 18*, 319-323.

Hotz, C., & Brown, K. (2004). Assessment of the risk of zinc defciency in populations and options for its control. *Food and Nutrition Bulletin* (15), 94-204.

Hussien, T., Bowden, R., Gill, B., Cox, T., & Marshal, D. (1997). Performance of four new leaf rust resistance genes transferred to common wheat from Aegilopes tauschi and Triticum monoccum. *Plant Disease , 81*, 582-586.

Initiative, m. (2009). *http://www.micronutrient.org/english/view.asp?x=1*.

Jenings, A., & Morton, R. (1963). Changes in carbohydrate, protein, and nonprotein nitrogenous compounds of developing wheat grain. *Australian Journal of Biological Sciences , 16*, 318.

Johnson, A., & McCaldin, D. (1958). Formation of yellow condensation products from amino compounds and ninhydrin. *Journal of Chemical Society , 158*, 817.

Kadowaki, K.-i. (1993). Organization and post-transcriptional modification of mitochondrial genes in higher plants. *Journal of Plant Research , 106*, 89-99.

Kerber, E., & Dyck, P. (1973). Inheritence of stem rust resistance transferred from diploid wheat (*Triticum monococcum*) to tetraploid and hexaploid wheat and chromosome location of the gene involoved. *Kanadian Journal of Genetic and Cytology , 15*, 397-409.

Kichey, T., Heumez, E., Pocholle, D., Pageau, K., Vanacker, H., Dubois, F., et al. (2006). Combined agronomic and physiological aspects of nitrogen management in wheat highlight a central role for glutamine synthetase. *New Phytologist , 169*, 265-278.

Kimball. (2009, 05). *http://users.rcn.com/jkimball.ma.ultranet/BiologyPages/P/Proteins.html*. (kimball, Editor) Retrieved from /jkimball.

Knox, R., Kohler, G., Palter, R., & Walker, H. (1970). Determination of tryptophan in feeds. *Analytical Biochemistry , 36*, 136-143.

Kutman, U. B., Yildiz, B., Ozturk, l., & Cakmak, I. (2010). Biofortification of durum wheat with zinc through soil and foliar application of nitrogen. *Cereal Chemistry , 87*, 1-9.

Lakhan, S., & Vieira, K. F. (2008). Nutritional therapies for mental disorders. *Nutrition Journal , 7:2*, 1-8.

Llames, C.R. & Fontaine, J. (1994). Determination of Amino Acids in Feeds: Collaborative Study. *Journal of AOAC international*, 77, 1362-1402.

Lamothe, P., & McCormick, P. (1973). Role of hydrindantin in the determination of amino acids using ninhydrin. *Analytical Chemistry , 45(11)*, 1906-1911.

Laskar, S., Sinhababu, A., & Hazra, K. (2001). A modified spray reagent for the detection of amino acids on thin layer chromatography plates. *Amino Acids , 21*, 201-204.

Lasztity, R. (1996). *The chemistry of cereal proteins.* (2nd Ed ed.). Florida: CRC Press Inc,.

Lill, J. R., Ingle, E. S., Liu, P. S., Pham, V., & Sandoval, W. N. (2007). Micowave-assisted proteomics. *Mass Spectrometery Reviews , 26*, 657-671.

Luis, F., Garcia, D. M., Yahia, R., Yanessa, M., & Conxita, R. (2007). Grain-protein content and amino acid composition of wheat composition in the grain of durum wheat grownunder different water and temperature regimes in mediterranean environment. *Journal of Agricultural and Food Chemistry , 66*, 8144–8151.

Lupano, C. E. (1994). Effect of heat treatment in very acidic conditions on whey proteins isolate properties. *Journal of Dairy Science , 77*, 2191-2198.

M, P. D. (1997). *Microwave Engineering* (second edition ed.). USA: John Wiley& SonsInc.

Marschner, H. (1995). *Mineral nutrition of higher plants.* London: Academic Press.

Mason, V., Rudemo, M., & Anderson, S. (1980). Hydrolysate preparation for amino acid determinations in feed constituents. *Tierphysiol Tierernahr Futtermittelkd , 43(1)*, 35-48.

Merrill AL, Watt BK. (1973). Energy value of food—basis and derivation. Washington, DC: *US Department of Agriculture Handbook* No. 74

Messia, M., Di Falco, T., Panfil, G., & Marconi, E. (2008). Rapid determination of collagen in meat-based foods bymicrowave hydrolysis of proteins and HPAEC–PAD analysis of 4-hydroxyproline. *Meat Science , 80*, 401-409.

Milling, H. (2007). *http://www.horizonmilling.ca/resources/wheat_kernel.shtml*.

Moore, S. (1968). Amino acid analysis: dimethyl slfoxide as a solvent for the ninhydrin reaction. *The Journal of Biological Chemistry , 243*, 6281-6283.

Moore, S., & Stein, W. (1954). A modified ninhydrin reagent for the photometric determination of amino acids and related compounds. *The Journal of Biological Chemistry , 211*, 907-913.

Moose, J. (1990). Nitrogen to protein conversion factor for ten cereals and six legumes or oilseeds. A reappraisal of its definition and determination. *The Journal of Agricultural and food Chemistry , 38*, 18-24.

Noltmann, E., Mahowald, T., & Kupy, S. (1962). Studies on adenosine triphosphate transphosphorulase. *Journal of Biological Chemistry , 237*, 1146-1154.

Olcott, H., & Fraenkel-Conrat, H. (1947). Formation and loss of cystine during acid hydrolysis of proteins. Role of tryptophan. *Journal Of Biological Chemistry , 171*, 583-592.

Osborne, T. (1907). The proteins of the Wheat Kernel. *Science , 26*, 865.

Ozaki, Y., Ohashi, T., & Kume, S. (1987). Potentiation of neutrophil function by recombinant DNA-produced interleukin 1a. *Journal of leukocyte biology , 42*, 621-627.

Ozman, A., Eichberg, J., Widgar, W., Voet, D., Voet, J., & Pratt, C. (2009). *Fundamentals of Biochemistry* (Third ed.). UK: John Wiley & Sons Inc.

Ozturk, L., Altintas, G., Erdem, H., Gokmen, A. O., Yazici, A., & Cakmak, I. (2009). Localization of iron, zinc, and protein in seeds of spelt (Triticum aestivum ssp. spelta) genotypes with low and high protein concentration. *The Proceedings of the International Plant Nutrition Colloquium*, (pp. 1-6). Sacremanto.

Ozturk, L., Yazici, M., Yucel, C., Torun, A., Cemal, C., Bagci, A., et al. (2006). Concentration and localization of zinc during seed development and germination in wheat. *Physiologia Plantarum , 128*, 144-152.

Palis, S., Kays, C., Deval, C., Bruht, A., Fafournoux, P., & Kilberg, M. (2009). Specificity of amino acid regulated gene expression: analysis of gene subjected to either complete or single amino acid deprivation. *Amino Acids , 37*, 79-83.

Payne, P. (1987). Genetics of wheat storage proteins and the effect of allelic variation on bread-making quality. *Annual Review of Plant Physiology , 38*, 141-153.

Pearson, J., & Rengal, Z. (1995). Uptake and distribution of Zn-65 and mn-54 in wheat grown at sufficient and deficient levels of Zn and Mn.2.2 during grain development. *Journal of Experimental Botany , 46*, 841-845.

Pecavar, A., Prosek, M., Fercej-Temeljotov, D., & Marsel, J. (1990). Quantitative evalution of amino acids using microwave accelerated hydrolysis. *Chromatographia , 30*, 159-162.

Peleg, Z., Saranga, Y., Yazici, A., Fahima, T., Ozturk, L., Budak, H., Pelg,Z.,Morgounov,A.,Fahima,T., Saranga,y., & Cakmak, I. (2007). Grain zinc, iron and protein concentrations and zinc-efficiency in wild emmer wheat under contrasting irrigation regimes. *Plant Soil*, 306, 57-67

Phani, A., & Santucci, S. (2006). Evaluation of structural and mechanical properties of aluminum oxide thin films deposited by a sol–gel process: Comparison of microwave to conventional anneal. *Journal of Non-Crystalline Solids , 352*, 4093–4100.

Picton, S., McCluskey, J., Flatt, P., & McClenaghan, N. (2002). Effects of cytotoxic agents on functional integrityand antioxidant enzymes in clonal beta cells. *Diabetes & Metabolism , 28*, 3S70-73S77.

Pierce. (2006). *http://www.funakoshi.co.jp/data/datasheet/PCC/29564.pdf*. (Pierce)

Piez, K. A., & Morris, L. (1960). A modified procedure for the automatic analysis of amino acids. *Analytical Biochemistry , 1*, 187-201.

Pozar, D. (1997). *Microwave Engineering* (second edition ed.). Willey.

Prasad, A. S. (2008). Zinc in human health: effect of zinc on immune cells. *Journal of Molecular Medicine , 14(5-6)*, 3 5 3 - 3 5 7.

Prasad, A., Bao, B., Beck, F., & O Kucuk, F. (2004). Antioxidant effect of zinc in humans. *Free Radical Biology & Medicine , 37*, 1182-1190 .

Prasad, A., Bao, B., Beck, F., & Sarkar, F. (2001). Zinc activates NF-κB in HUT-78 cells. *Journal of Laboratory and Clinical Medicine , 138*, 250-256.

Prasad, A., Fitzgerald, J., Hess, J., Kaplan, J., Pelen, F., & Dardenne, M. (1993). Zinc deficiency in elderly patients. *Nutrition , 9(3)*, 218-224.

Prasad, A., Meftah, S., Abdallah, J., Kaplan, J., Brewer, G., Bach, J., et al. (1988). Serum thymulin in human zinc deficiency. *Journal of Clincal Investigation , 82(4)*, 1202-1210.

Prasnna, B., Vasal, S., Kassahun, B., & Singh, N. (2001). Quality protein maize. *Current Science , 81*, 1308-1319.

Rachon, L., & Shumi, G. (2009). Comparison of chemical composition of selected winter wheat species. *Journal of Elementology , 14*, 135-146.

Ranum, P. (2001). Solving micronutrient deficiency problems. *Cereal food World , 46*, 441-443.

Rawn, D. J. (1989). *Biochemeistry.* Burlington, North carolina, USA: Neil Patterson Publishers.

Rhoads, m., & G, W. (2009). Glutamine, arginine, and leucine signaling in the intestine. *Amino Acids , 37*, 111-122.

Rhoads, m., & G, W. (2009). Glutamine, arginine, and leucine signaling in the intestine. *Amino Acids , 37*, 111-122.

Roach, D., & Gehrke, C. (1970). The hydrolysis of proteins. *Journal of Chromatography , 52(3)*, 393-404.

Roth, M. (1971). Fluorescence reaction for amino acids. *Analytical Chemistry , 43(7)*, 880-882.

Ruhemann, S. (1910). Triketohydrindene hydrate. *Journal of the Chemical Society, Transactions , 97*, 2025-2031.

Shewry, P. r. (2007). Improving the protein content and composition of cereal grain. *Journal of Cereal Science , 46*, 239-250.

Shewry, P. R., & Halford, N. G. (2002). Cereal seed storage proteins: structure, properties and role in grain utilization. *Journal of Experimental Botany , 53*, 947-958.

Shewry, P. (2009). Wheat. *Journal of Experimental Botany , 60*, 1537-1553.

Shu, N., Zhou, T., & Hovmeller, S. (2008). Prediction of zinc-binding sites in proteins from sequence. *Bioinformatics , 24*, 775-782.

Simmonds, D. (1978). Structure, compostion and biochemistry of cereal grains. *Cereals* , 78, 105-137.

Simmonds, D. (1981). Wheat proteins: their chemistry and nutritional potential. *Wheat Science* , 32, 149-157.

Spackman, D., Stein, W., & Moore, ,. (1958). Chromatography of amino acids on sulphonated polystyrene resins. An improved system. *Analatical Chemistry , 30*, 1185-1190.

Spurway, R. (2008, 3 10). *How is grain protein formed*. Retrieved from http://www.regional.org.au/au/roc/1988/roc198823.htm: http:www.regional.org.au/au/roc/1988/roc198823.htm?print=1

Suryawan, A., O`Connor, P., Bush, J., Nguyen, H. V., & Davis, T. (2009). Differential regulation of protein synthesis by amino acids and insulin in peripheral and visceral tissues of neonatal pigs. *Amino Acids , 37*, 97-104.

Suryawan, A., O`Connor, P., Bush, J., Nguyen, H. V., & Davis, T. (2009). Differential regulation of protein synthesis by amino acids and insulin in peripheral and visceral tissues of neonatal pigs. *Amino acids , 37*, 97-104.

Tamis, c., Boglarka, K., Rakszegi, M., Wilkinson, M. d., Yang, M.-S., Lang, L., et al. (2009). Transgenic approach to improve wheat (Triticum aestivum L.) nutritinal quality. *Plant Cell Reports , 28*, 1085-1094.

Tea, I., Genter, T., Naulet, N., Lummerzheim, M., & Kleibe, D. (2007). Interaction between nitrogen and sulfur by foliar application and its effects on flour bread-making quality. *Journal of the Science of Food and Agriculture , 87*, 2853-2859.

Thompson, J., & Morris, C. (1959). Determination of amino acids from plants by paper chromatography. *Analatical Chemistry , 31*, 1031-1037.

Torrance, J., MacArthur, M., & Thornton, J. (2008). Evolution of binding sites for zinc and calcium ions playing structural roles. *Proteins-Structure Function and Bioinformatics , 71*, 813-830.

Troccoli, A., Borrelli, G., Vita, D., C, F., & Di Fonzo, N. (2000). Durum Wheat Quality: A multidisciplinary concept. *Journal of Cereal Science , 32*, 99-113.

Tyler, M. I. Amino Acid Analysis . In *Amino Acid Analysis Protocols* (Vol. 159, pp. 1-7). Humana Press.

Uauy, C., Distelfed, A., Fahima, T., Belchl, A., & Dubcovsky, J. (2006). A NAC Gene Regulating Senescence Improves Grain protein, Zinc. and Iron Content in wheat. *Science , 314*, 1298 – 1301.

Valkoun, J., Kucerova, D., & Bartos, P. (1986). Transfer of leaf rust resistance from Triticum monococcum L. to hexaploid wheat. *Zeitschrift Fur Pflanzenzuchtung , 96*, 271-278.

Vogel, K., Johnson, V., & Mattern, P. (1978). Protein and lysine contents of endosperm and bran of the parents and progenies of crosses of common wheat. *Crop Science , 18*, 751-754.

Von Wiren, N., Klair, S., Bansal, S., Briat, J., Khodr, H., Shioiri, T., et al. (1999). Nicotianamine chelates both Fe-III and Fe-II Implications for metal transport in plants. *Plant Physiology , 119*, 1107-1114.

Walker, C., Ezzati, M., & Black, R. (2009). Global and regional child mortality and burden of disease attributable to zinc deficiency. *European Journal of Clinical Nutrition , 63*, 591-597.

Walsh, C. T., Sandstead, H. H., Prasad, A. S., Newberne, P. M., & Fraker, P. J. (1994). Zinc: health effects and research priorities for the 1990s. *Environmental Health Perspectives , 102(2)*, 5-46.

Wang, W., Qiao, S., & Li, D. (2009). Amino acids and gut function. *Amino Acids , 37*, 105-110.

Wang, Y., Khan, K., Hareland, G., & Nygaed, G. (2007). Distribution of protein composition in bread wheat flour mill streams and relationship to breadmaking quality. *Cereal chemistry , 84*, 271-275.

Weiss, M., Manneberg, M., Jranville, J., Lahm, H.-W., & Fountoulakis, M. (1998). Effect of hydrolysis method on the determination of the amino acid composition of proteins. *Journal of Chromatography , 995*, 263-275.

Weiss, M., Manneberg, M., Jranville, J., Lahm, H.-W., & Fountoulakis, M. (1998). Effect of hydrolysis method on the determination of the amino acid composition of proteins. *Journal of Chromatography , 995*, 263-275.

Welch, R. (1986). Effects of nutrient deficiencies on seed production and quality. *Plant Nutrition , 2*, 205-247.

Welch, R., & Graham, R. (1999). A new paradigm for world agriculture: meeting human needs- productive, sustainable, nutritious. *Field Crops Research , 60*, 1-10.

Welch, R., & Graham, R. (2004). Breeding for micronutrients in staple food crops from a human nutrition perspective. *The Journal of Experimental Botany* (55), 353-364.

White, P. j., & Broadley, M. R. (2009). Biofortification of crops with seven mineral elements often lacking in human diets – iron, zinc, copper,calcium, magnesium, selenium and iodine. *New Phytologist , 182*, 49-84.

World health Organization, Meeting of the intersted Parties:Nutrition. (2001). Retrieved from http://www.who.int/mipfiles/2299/mip_01_APR_SDE_3.en.pdf.

Yao, G., Qi, D., & Zhang, X. (2008). Functionalized magnetic carbonaceous microspheres for trypsin immobilization and the application to fast proteolysis. *Journal of Chromatography , 1215*, 82-91.

Young, v. R., & Pellet, p. L. (1985). Wheat protein in relation to protein requirments and availability of amino acids. *the American Journal Of Clinical Nutrition , 41*, 1077-1090.

Zhao, J., Sua, Y., Dunhama, S., rakszegib, M., Bedo b, Z., McGrath a, S., et al. (2009). Variation in mineral micronutrient concentrations in grain of wheat lines of diverse origin. *Journal of Cereal Science , 49*, 290-295.

Zhong, H., Marcus, S. L., & Li, l. (2005). Microwave-assisted acid hydrolysis of proteins combined with liquid chromaography MALDI MS/MS for protein identification. *Journal of the American Society for Mass Spectrometry , 16*, 471-481.

Zumwalt, R., Absheer, J., Kaiser, F., & Gehrke, C. (1987). Acid hydrolysis of proteins for chromatographic analysis of amino acids. *Journal of the Association of Official Analytical Chemists , 70(1)*, 147-151.

APPENDIX A

Chemicals and Buffers

Absolute ethanol	Riedel	32221
Hydrochloric acid (37%)	Merck	100314
Hydrogen peroxide (30 %)	Merck	107209
Isopropanol	Merck	1.09634
Nitric acid (65%)	Merck	1.00456
Phenol	Merck	1.00206
Sodium hydroxide	Merck	1.06495
Sulfuric acid (98%)	Merck	1.00748
Sodium disulfite	Merck	1.06528
Formic acid (98%)	Merck	1.00264
Niterogen gas (N2)	Linde	
Amino acid standard (AAS018)	Sigma	029K0705
l-Cystiec acid	Sigma	30170
L-Ornithine	Sigma	078k0732
L-Methionine sulphone	Sigma	135G458
Methionine sulphoxide	Fluka	64430
L-Tryptophan	Sigma	93659
Sodium citrate loading buffer (pH=2.2)	Biochrom	
Sodium citrate loading buffer (pH=2.65)	Biochrom	
Sodium citrate loading buffer (pH=3.35)	Biochrom	
Sodium citrate loading buffer (pH= 4.25)	Biochrom	
Sodium citrate loading buffer (pH=8.6)	Biochrom	
Ninhydrin solution	Biochrom	
Ultrasolve plus	Biochrom	

APPENDIX B

Equipments

Distilled water	Millipore,Elix-S, Fance,
Milling machine	Pulverisette 9, Fritsch GmbH, Idar-Oberstein, Germany
PH meter	HANNA
CEM MARS Express microwave	CEM MARS Corporation, USA
Biochrom 30 amino acid analyzer	Biochrom Inc, England
Inductively coupled plasma-optical Emission spectroscopy (ICP-OES)	Varian, Vista-Pro ccd, Australia
Magnetic stirrer	IKA-Werke, Germany
Micro liter pipette	Eppendorf, Germany
Balance	Denver instrument
Ice machine	Scotsman Inc., AF20, USA
N measure	Leco Truspec CN, USA
Oven	Memmert D06062, Germany
Refrigerator	+4 Arçelik, Turkey