Research Article

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MEDITERRANEAN AGRICULTURAL SCIENCES
(2024) 37(3): 129-136
DOI: 10.29136/mediterranean.1551068

Quantification and comparison of gliadin proteins in ancient wheats grown in rainfed and irrigated conditions

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ARTICLE INFO

Received: September 17, 2024
Received in revised form: October 23, 2024
Accepted: October 24, 2024

***Keywords*:**

Ancient wheat
BCA Assay
Gliadin
Protein determination
Wheat extraction

ABSTRACT

Protein quantification is crucial for assessing the nutritional and functional qualities of wheat. This study quantified the gliadin content, a major component of wheat storage proteins, in 24 wheat genotypes, including ancient varieties such as einkorn (*Triticum monococcum*), emmer *(Triticum dicoccum)*, and spelt *(Triticum spelta).* These varieties were cultivated under rainfed and irrigated conditions in the Konya/Ilgın region of Türkiye. Gliadin proteins were extracted using a 70% ethanol solution to isolate the soluble fractions, which were subsequently analyzed using the Bicinchoninic Acid (BCA) assay. The results revealed significant variation in gliadin content among the genotypes. In general, samples grown under irrigated conditions exhibited higher protein concentrations compared to those grown under rainfed conditions. Among the varieties, Karahan (16339.07 µg mL^{-1}) and Soana (15826.99 µg mL^{-1}) had the highest protein contents under irrigated and rainfed conditions, respectively. These findings demonstrate the impact of both environmental and genetic factors on protein composition, highlighting the importance of ancient wheat varieties in sustainable agriculture and their potential to enhance modern dietary nutrition.

1. Introduction

Food security and nutritional quality have become increasingly critical in the global agriculture landscape. Among staple crops, wheat remains one of the most important worldwide, serving as a primary dietary source for billions across diverse cultures (Shewry and Hey 2015).Within the broad spectrum of wheat varieties, ancient types have garnered renewed attention due to their health benefits and resilience to environmental stressors (Dinu et al. 2018).

Ancient wheat types such as einkorn (*Triticum monococcum*), emmer (*Triticum dicoccum*), and spelt (*Triticum spelta*) have rich genetic backgrounds and are often distinguished by their lower gluten content, higher micronutrient levels, and greater resilience to stress conditions, such as drought (Shewry 2009). Historically they were cultivated in regions that are now part of modern Türkiye, which is known for its diverse climatic conditions and agricultural practices. The growing interest in ancient wheat varieties is motivated by their potential contributions to sustainable agriculture and human health, particularly in light of rising awareness of gluten-related disorders and the benefits of whole grains (Shewry and Hey 2015).

Mature wheat grains contain approximately 8%–20% proteins, comprising 15%–20% water-soluble albumins and globulins, while the remaining 80%–85% is made up of water-insoluble glutenins and gliadins (Zilić et al. 2011). Glutenins dissolve in dilute acetic acid, while gliadins dissolve in 70% alcohol (Osborne 1924). Due to their prominent levels of proline and glutamine residues, glutenins and gliadins are collectively termed prolamins.

These proteins are not only critical for assessing the nutritional quality of wheat but also for understanding its functional properties, especially in bread-making. Gluten, formed by the interaction between glutenin and gliadin, significantly influences dough elasticity and strength, making protein composition a key factor in evaluating wheat varieties for baking potential (Thanhaeuser et al. 2015). In a related study, 49 emmer lines, 36 einkorn lines, and 3 modern durum wheat cultivars (controls) were analyzed, with specific einkorn lines showing the highest protein levels for these varieties (Akar et al. 2019).

Gliadin proteins play a fundamental role in forming the gluten network and contribute to the nutritional value of wheat, making their accurate quantification vital for both health and food science applications (Thanhaeuser et al. 2015). One commonly used method for quantifying gliadin is the Bicinchoninic Acid (BCA) assay, valued for its simplicity, high sensitivity, and reproducibility (Walker 1994).

In addition to the BCA assay, the Dumas and Kjeldahl methods are widely used for protein quantification though they are based on different principles. The Dumas method, which involves combustion to determine the nitrogen content of a sample, is rapid and requires minimal reagents, making it suitable

for high-throughput analyses. It also offers an advantage over the Kjeldahl method due to its eco-friendly nature, as it does not require hazardous chemicals such as sulfuric acid. In contrast, the Kjeldahl method is a wet-chemical technique that involves digesting the sample in sulfuric acid, followed by neutralization and distillation to quantify nitrogen. Though labor-intensive, it remains a gold standard for protein determination due to its high accuracy and applicability to a wide range of sample types, including agricultural products. Together, these methods provide complementary insights, especially when analyzing agricultural samples such as wheat. Used alongside the BCA assay, they offer a comprehensive understanding of the total protein and specific protein components such as gliadins and glutenins.

The BCA assay quantifies proteins by reducing Cu^{2+} ions in an alkaline solution to Cu^{1+} ions, in a reaction such as the biuret method but with greater sensitivity. In this assay, proteins reduce Cu^{2+} to Cu^{1+}, which subsequently forms a purple complex with two molecules of bicinchoninic acid (BCA). The complex absorbs light at 562 nm, and the absorbance is directly proportional to the protein concentration in the sample (Smith et al. 1985; Walker 1994).

The BCA assay is favored for its compatibility with various detergents and reducing agents, which can interfere with other protein assays, such as the Bradford assay (Walker 1994; Wiechelman et al. 1988). Its application in wheat protein quantification, particularly for gliadin, provides critical insights into wheat's nutritional and functional properties. By comparing sample absorbance to a standard curve generated from known protein concentrations, accurate quantification of gliadin content can be achieved (Schoel et al. 1995)

Understanding the protein composition of ancient wheat varieties, particularly gliadins, is crucial for evaluating both their nutritional quality and functional capacity in food applications. The BCA assay, in conjunction with methods such as the Dumas and Kjeldahl methods, offers reliable and sensitive tools for this purpose. As interest in ancient wheat varieties grows due to their potential benefits for human health and sustainable agriculture, robust protein quantification methods will play a pivotal role in deepening our understanding of these grains.

The aim of this study was to determine and compare the gliadin protein contents of ancient wheat varieties grown under rainfed and irrigated conditions. Samples were collected from Ilgın/Konya Türkiye and analyzed by BCA assay.

2. Materials and Methods

2.1. Sample Collection and Preparation

The study utilized a trial set of 24 wheat genotypes, which included a variety of candidates from the ancient cultivated species einkorn (*Triticum monococcum*) and emmer (*Triticum dicoccum*). The genotypes also comprised local bread wheat varieties selected through breeding, as well as varieties developed both before and after the Green Revolution (pre- and post-1970). Detailed information on the genotypes used in the study is presented in Table 1.

Table 1. Characteristics of the genetic materials used in the study

Genotypes	Breeding Methods, Registration Years, and Classification	Pedigree
Songar* (Einkorn)	Historical/selection/2019 pre-green revolution	Selection from local population
Mokan* (gernik)	Historical/selection/2019 pre-green revolution	Selection from local population
Ak702	Local variety/selection/1931 pre-green revolution	A mixture of two pure lines obtained from Akbuğday grown around Eskişehir
Sertak	Local variety/selection/1936 pre-green revolution	A mixture of two pure lines numbered 1721 and 1731
Yektay	Old variety/crossbreeding/1968 pre-green revolution	Selection from San Marino wheat population
Bezostaja1	Old variety/crossbreeding/1968 pre-green revolution	LUTESCENS-17/ SKOROSPELKA-2
Bolal	Old variety/crossbreeding/1970 pre-green revolution	CHEYENNE//KENYA-324/MENTANA
Gerek79	Old variety/crossbreeding/1979 post-green revolution	MENTANA/MAYO-48//4-11/3/YAYLA-305
Kırkpınar	Old variety/crossbreeding/1979 post-green revolution	HYSLOP/SIETE-CERROS-66
Sultan95	Old variety/crossbreeding/1995 post-green revolution	AGRI/NACOZARI-76
İkizce96	Old variety/crossbreeding/1996 post-green revolution	ARTHUR*2/SIETE-CERROS-66//BRILL
Pehlivan	Old variety/crossbreeding/1998 post-green revolution	BEZOSTAYA-1/TEVERE/5/CENTRIFEN/BEZOSTAYA-1//SUWEON-92/CI-13645/3/NAINARI-60/4/(SIB)EMU
Karahan	Old variety/crossbreeding/1999 post-green revolution	C-126-15/COLLAFEN/3/NORIN-10-BREVOR/P-14//(P101)PULLMAN-101/4/KIRAC-66
Alparslan	New variety/crossbreeding/2001 post-green revolution	TX-69-A-509-2//BBY2/FOX
Nenehatun	New variety/crossbreeding/2001 post-green revolution	NORD DEPREZ/PULLMAN SELECTION 101//BLUEBOY
Sönmez2001	New variety/crossbreeding/2001 post-green revolution	BEZ//BEZ/TVR/3/KREMENA/LOV29/4/KATIA1
Konya2002	New variety/crossbreeding/2002 post-green revolution	KANRED/ TENMARQ//P-211-6/3/2183/ CO-652643/LANCER
Tosunbey	New variety/crossbreeding/2004 post-green revolution	ECVD-12/KIRAC-66//(SIB) CROW
Ahmetağa	New variety/crossbreeding/2004 post-green revolution	F-885-K-1-1/SIOUXLAND
Esperia	New variety/crossbreeding/2011 post-green revolution	B-16-3/LINEA-RUSSA
Bora	New variety/crossbreeding/2014 post-green revolution	H-31/TRAP-1-F-2//ENESCO
Genesi	New variety/crossbreeding/2014 post-green revolution	COLFIORITO/HEREWARD
Soana	New variety candidate/crossbreeding/2019 post-green revolution	unknown
Galinda	New variety candidate/crossbreeding/2019 post-green revolution	AHMETAĞA/ESPERIA/ //ESPERIA

The wheat samples were cultivated during 2022-2023 in the Ilgın/Konya region of Türkiye, following a randomized block design with three replications. Samples from ancient wheat varieties were collected from both rainfed and irrigated fields, allowing for a comprehensive analysis of the impact of irrigation on protein content and composition. The wheat grains were harvested at maturity, cleaned, dried, and stored under controlled conditions to minimize degradation. Following standard procedures, the wheat grains were milled into flour to ensure uniformity for subsequent analysis.

2.2. Chemicals, consumables and instrumentation

All chemicals used in the study were of analytical and/or liquid chromatographic grade. Ethanol was sourced from Isolab (Wertheim, Germany). The water for dilutions and preparation of samples was provided by an ultrapure water purification system from MP Minipure (Ankara, Türkiye). Protein extraction was conducted using a Biosan TS-100 type shaker. To determine the constant protein amount, the Pierce BCA Assay Kit (Thermo Fisher Scientific) was used. Absorbance measurements were taken at Thermo Scientific Multiskan GO Microplate Spectrophotometer.

2.3. Protein extraction

For wheat flour extraction, 20 grams of wheat grains were ground and separated into flour and bran to provide sufficient material for the experiments. Initial trials showed that the parameters were optimal for dissolving gliadin proteins at 30°C for 1.5 hours.

According to the protocol (Malalgoda et al. 2018) approximately 250 mg of each flour sample was weighed into sterile 1.5 mL Eppendorf tubes. Then, 750 µL of 70% ethanol was added. Extraction was started at 1400 rpm and 30°C for 1.5 hours on a thermoshaker. After extraction, the samples were then centrifuged at 4550 rpm for 15 minutes. The supernatant containing gliadin was filtered through a 0.45 µm filter into a new tube. Three replicates were prepared for each sample and the extracts were obtained in the same tubes at -18°C. To prevent extract loss during centrifugation, each Eppendorf tube was wrapped in parafilm. Replicates were performed for each sample during extraction, and the replicates were included in the BCA protein analysis in the same manner.

Since multiple measurements were required for determining the protein content in wheat, the multi-sample measurement technique using the microplate procedure was applied.

2.4. BCA protein analysis

The BCA Protein Assay is a widely used method for quantifying total protein content. Pierce BCA Protein Assays have a unique benefit over the Coomassie dye-based methods (Bradford). The methods are compatible with samples that contain up to 5% surfactants and are assumed much less by protein compositional variations, providing greater protein to protein uniformity. The BCA assay was performed according to established protocols, with various concentrations of a bovine serum albumin (BSA). Spectrophotometric measurements were conducted at 562 nm, correlating absorbance values to the protein concentration in the wheat samples. To prepare the protein standard, a 2 mg mL^{-1} albumin was diluted, and each 1 mL was sufficient to prepare the necessary standard. The prepared standards and sample extracts were placed on a microplate for measurement. Extracts containing Gliadin were diluted 50-fold, and then 25 µL of each was added to the microplate wells, followed by 200 µL of reactive BCA agent. The final concentrations of BSA for the standard curve, prepared with different volumes of ultrapure water, are shown in Table 2.

For each standard concentration, 25 µL of the diluted standard was pipetted into a microplate well, and 200 µL of reactive agent was added. The plate was incubated at 37°C for 30 minutes. Absorbance measurements were taken at 562 nm with a spectrophotometer. The plate was shaken for 30 seconds and then incubated at 37°C for 30 minutes before measuring the absorbance at 562 nm. All samples were measured in triplicate, and a standard curve graph (Figure 1) was created based on the average OD values. Both rainfed and irrigated trials were analyzed, and post-harvest physicochemical and rheological properties of the grain products from each plot were determined, as specified in the project proposal.

2.5. Statistical analysis

All chemical analyses were carried out in three replicates per plot and the results obtained were analyzed statistically. Significant differences between genotypes were analyzed by the ANOVA using the Minitab 17.0 statistical program. A test was performed to evaluate the significance of differences between the species means. Differences with $P<0.05$ were considered significant in both tests. The statistical analysis, particularly the ANOVA results, revealed significant differences in gliadin concentrations across both wheat varieties and cultivation conditions at a 1% significance level.

3. Results and Discussion

Gliadin, one of the key proteins in wheat that forms gluten, is essential for understanding the functional properties of flour, particularly in dough quality and elasticity (Shewry et al. 2002). The analysis revealed significant differences in gliadin concentrations between varieties, as well as between cultivation conditions. In the study, using Bicinchoninic Acid (BCA) assay, gliadin concentrations were quantified for 24 different ancient and modern wheat varieties grown under both rainfed and irrigated conditions. The results indicated that ancient wheat varieties, such as Einkorn and Gernik, exhibited higher gliadin concentrations, compared to modern wheat varieties. Notably,

Table 2. Results of BCA protein quantification and albumin standard measurement

Vial	Dilution Volume (µL)	BSA Standard Volume (µL)	Final Con. (µg mL^{-1})
A	0	300 µL BSA Stock	2000
B	125	375 µL BSA Stock	1500
C	325	325 µL BSA Stock	1000
D	175	175 µL from Diluted Vial B	750
E	325	325 µL from Diluted Vial C	500
F	325	325 µL from Diluted Vial E	250
G	325	325 µL from Diluted Vial F	125
H	400	100 µL from Diluted Vial G	25

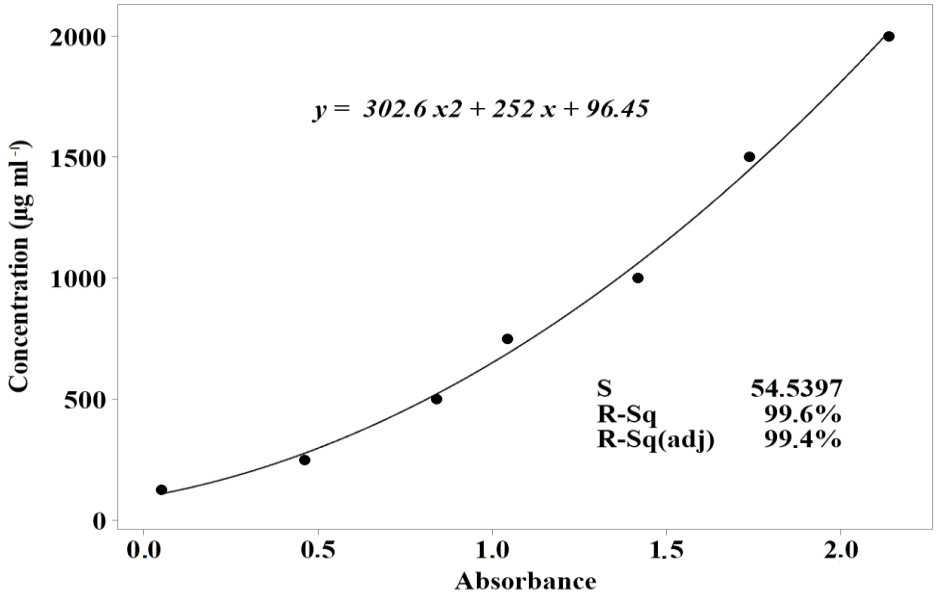

Figure 1. BSA standard concentration.

the Karahan variety, a more recent wheat genotype, displayed the highest gliadin concentration across both cultivation conditions, suggesting its potential use in high-protein applications such as bread-making. Surprisingly, despite their historical reputation for higher nutrient content, ancient varieties such as Einkorn showed moderate gliadin levels. (Dinu et al. 2018).

3.1. Gliadin concentration in ancient and modern wheat varieties

The gliadin concentrations in various ancient and modern wheat genotypes were quantified using the Bicinchoninic Acid (BCA) assay. The gliadin concentrations for the 24 wheat genotypes grown under irrigated conditions are illustrated in the interval plot of gliadin concentration (irrigated varieties) (Figure 1). The data were analyzed for both cultivation environments: irrigated and rainfed. Gliadin, a key protein involved in gluten formation, is a critical component in determining the quality of wheat flour for bread-making. The concentration of gliadin was compared between the different genotypes to assess the impact of both genotype and cultivation conditions on protein levels.

The results, presented in Figure 1, revealed substantial variability in gliadin content across the different genotypes. The Alparslan variety exhibited the highest gliadin concentration at approximately 16946 µg mL^{-1}, while Sönmez2001 had the lowest gliadin concentration at 10745 µg mL^{-1}. Among ancient wheat varieties, Einkorn had a higher gliadin concentration compared to other ancient varieties such as Yektay, Bezostaja1, Bolal, Gerek79, Kırkpınar, Sultan95, İkizce, and Pehlivan under irrigated conditions.

Einkorn's higher gliadin levels among older cultivars, especially under irrigated conditions, can be attributed to its genetic heritage and historical adaptation to less favorable growing conditions. When grown with regular irrigation systems, Einkorn can have an improved ability to synthesize considerable amounts of protein compared to other varieties that are selectively bred for different purposes.

Both Karahan and Alparslan, classified as old varieties developed through crossbreeding, stand out for their high gliadin concentrations. These varieties, developed after the Green Revolution (post-1970), exemplify how modern wheat breeding can benefit from the robustness of ancient varieties to enhance protein content. Karahan had a notable gliadin concentration (16339.07 µg mL^{-1}), making it one of the top performers. In contrast, varieties such as Bezostaja1, Bolal, and Gerek79, also developed through crossbreeding methods, displayed comparatively lower gliadin levels.

The results of the gliadin concentrations under rainfed conditions (Figure 2) provide further insights into the adaptability and protein synthesis capabilities of various wheat genotypes.

The interval plot of gliadin concentration for rainfed varieties (Figure 3) illustrates that gliadin concentrations were generally lower under rainfed conditions compared to irrigated conditions. On average, the gliadin concentration across all varieties decreased by approximately 15% under rainfed conditions. This trend aligns with existing research that highlights the importance of water availability in protein synthesis in wheat. Water stress can hinder nutrient uptake and metabolic activities essential for protein formation (Blum 2011).

Despite the overall reduction, certain varieties maintained high gliadin concentrations under rainfed conditions. Notably, Alparslan and Karahan recorded gliadin concentrations of 16946 µg mL^{-1} and 16343 µg mL^{-1}, respectively, values close to their levels under irrigated conditions, indicating a strong resilience to water stress. Alparslan, registered in 2001, is a new variety developed through crossbreeding post-Green Revolution. Its pedigree is TX-69-A-509-2//BBY2/FOX. The breeding methods utilized advanced selection techniques focusing on both yield and stress tolerance, which likely contributed to its high gliadin content even under rainfed conditions. Karahan registered in 1999 as an old variety but developed post-Green Revolution through crossbreeding. Its complex pedigree (C-126-15/COLLAFEN/3/NORIN-10-BREVOR/P-14//(P101) PULLMAN -101/4/KIRAC-66) includes contributions from drought-resistant lines such asNorin-10. This genetic background may enhance its ability to sustain protein synthesis under limited water availability (Lumpkin 2015).

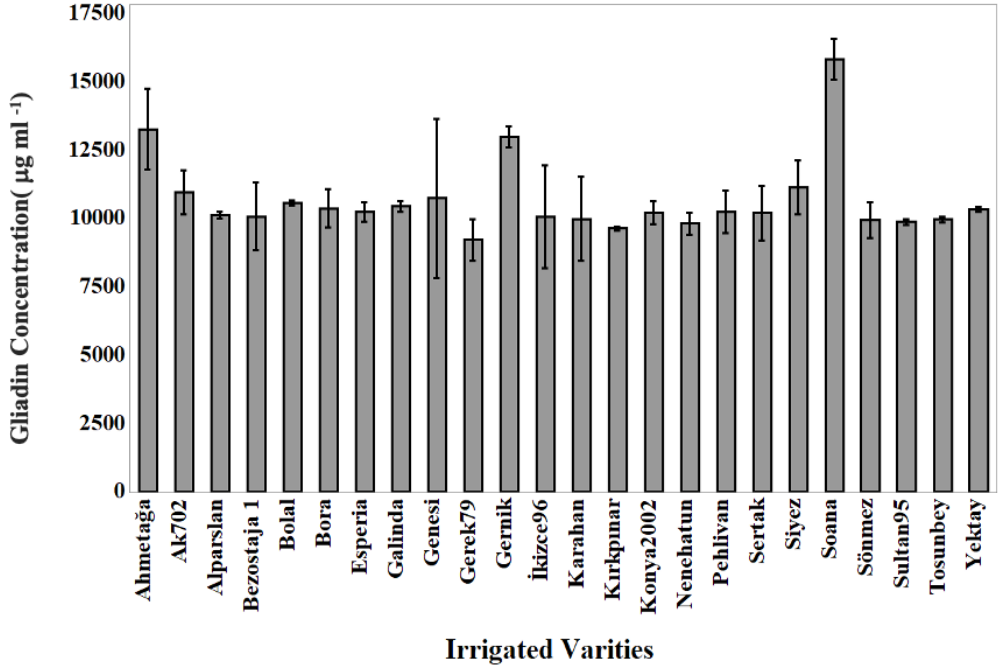

Figure 2. Interval plot of gliadin concentration (irrigated varieties).

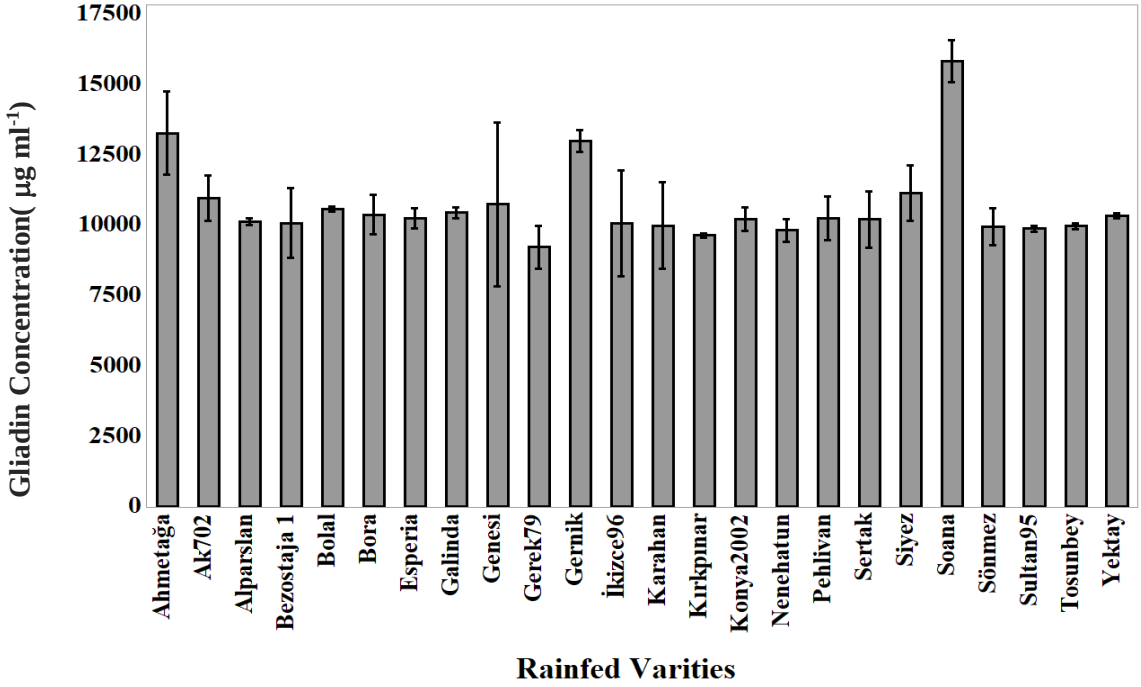

Figure 3. The interval plot of gliadin concentration (rainfed varieties).

Conversely, Sönmez2001, a new variety also registered in 2001 and developed through crossbreeding, displayed the lowest gliadin concentration at 10745 µg mL^{-1} under rainfed conditions. Its pedigree (BEZ//BEZ/TVR/3/KREMENA/LOV29/4/KATIA1) may lack specific traits associated with drought tolerance, making it more susceptible to water stress in terms of protein synthesis. Interestingly, the ancient variety Soana demonstrated a significant gliadin concentration of 13696 µg mL^{-1} under rainfed conditions, outperforming many modern varieties. Soana is classified as a new variety candidate registered in 2019 and developed through crossbreeding post-Green Revolution, with a pedigree of AQUILANTE/SOLEHIO.

The success of varieties such as Alparslan and Karahan demonstrates the potential of modern breeding to achieve high protein content even under sub-optimal conditions such as water scarcity.

The Interval plot comparing irrigated and rainfed conditions (Figure 4) demonstrates that gliadin concentrations were significantly higher under irrigated conditions than under rainfed conditions. The mean gliadin concentration for irrigated varieties was approximately 12500 µg mL^{-1}, while that for rainfed varieties was around 11500 µg mL^{-1}. This statistically significant difference (P<0.05) highlights the importance of water availability in enhancing protein accumulation in wheat.

The grouping information from the Tukey test (Table 3 and Table 4) provided additional statistical insights into the differences between genotypes. Genotypes such as Alparslan, Karahan, and Ahmetağa were consistently grouped in the highest category (Group A), whereas Sönmez2001 was placed in the lowest group (Group G) across both conditions. This clear separation of genotypes is based on their gliadin concentrations, with significant differences observed between groups.

The post-hoc Tukey test further validated these differences, with varieties such as Karahan and Alparslan consistently grouped into higher gliadin concentration categories, while varieties such as Bezostaja1 and Tosunbey were grouped into lower categories. This highlights the distinct protein profiles of wheat varieties studied, underscoring the influence of their genetic backgrounds and breeding histories.

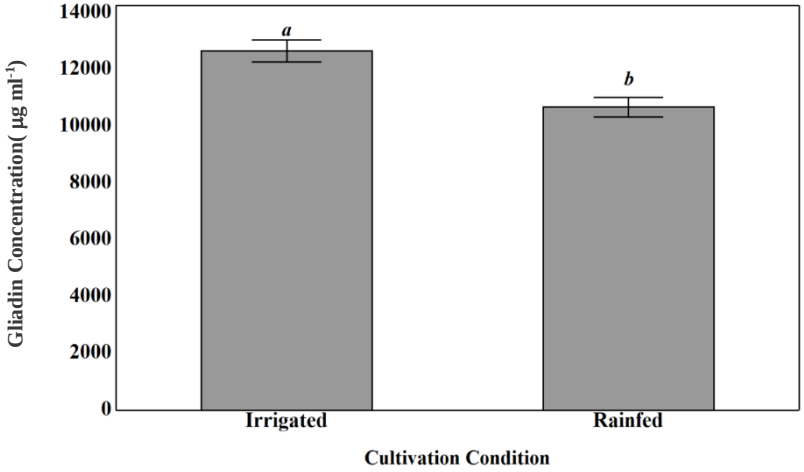

Figure 4. The interval plot comparing irrigated and rainfed conditions.

Table 3. Irrigated varieties grouping information using the Tukey method and 95% confidence

Varieties	Mean	Grouping					
Alparslan	16946±717	A					
Karahan	16343±595	A					
Ahmetağa	15646±190	A					
Einkorn	13714±137		B				
Soana	13697±145		B				
Kırkpınar	13115±154		B	C			
Pehlivan	12936±116		B	C	D		
Konya2002	12758±134		B	C	D	E	
Galinda	12565±095		B	C	D	E	F
Gerek79	12524±225		B	C	D	E	F	G
Sultan95	12443±398		B	C	D	E	F	G
Tosunbey	12343±109		B	C	D	E	F	G
Ak702	12341±051		B	C	D	E	F	G
Gernik	12268±261		B	C	D	E	F	G
İkizce96	11770±651			C	D	E	F	G
Esperia	11749±315			C	D	E	F	G
Genesi	11735±484			C	D	E	F	G
Bezostaja1	11631±126			C	D	E	F	G
Yektay	11580±171			C	D	E	F	G
Bolal	11429±167				D	E	F	G
Bora	11306±258					E	F	G
Sertak	11186±058					E	F	G
Nenehatun	11016±627						F	G
Sönmez2001	10745±979							G

Table 4. Rainfed varieties grouping information using the Tukey method and 95% confidence

Varieties	Mean	Grouping				
Soana	15828±298	A				
Ahmetağa	13267±595		B			
Gernik	12992±152		B			
Einkorn	11135±397			C		
Ak702	10965±328			C	D	
Genesi	10745±117			C	D	E
Bolal	10576±034			C	D	E
Galinda	10441±078			C	D	E F
Bora	10376±280			C	D	E F
Yektay	10341±034			C	D	E F
Esperia	10253±144			C	D	E F
Pehlivan	10243±314			C	D	E F
Konya2002	10220±171			C	D	E F
Sertak	10208±403			C	D	E F
Alparslan	10130±047			C	D	E F
Bezostaja1	10078±503			C	D	E F
İkizce96	10078±758			C	D	E F
Karahan	9987±620			C	D	E F
Tosunbey	9964±043			C	D	E F
Sönmez2001	9944±263			C	D	E F
Sultan95	9878±309			C	D	E F
Nenehatun	9821±163				D	E F
Kırkpınar	9635±032					F
Gerek79	9226±307					F

4. Conclusion

The results obtained revealed significant variation in protein content among ancient wheat varieties, with notable differences between rainfed and irrigated growing conditions. The investigation into protein quantification in ancient wheat varieties collected from Türkiye under contrasting cultivation conditions underscores the significance of both genetic diversity and environmental influences on agricultural outputs. The application of the BCA assay provides a reliable method for protein quantification, highlighting its utility in assessing the nutritional quality of ancient grains. As the global agricultural landscape evolves, the preservation and promotion of ancient wheat varieties may play a key role in addressing food security, enhancing human health, and fostering sustainable agricultural practices. This study emphasizes the critical importance of detailed agricultural and nutritional informing strategies for the utilization and conservation of these invaluable genetic resources.

Acknowledgments

The authors thank to the Turkish National Science Foundation (TUBITAK) which provided financial support for this research under grant number 123O333.

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