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Determination and Comparison of Hydrogen Bonds by STA and FT-Raman Spectroscopy in Gluten of Two Wheat Varieties

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Abstract

In the secondary structure of protein, peptide chains folded into a uniquely defined configuration, in which it is held by hydrogen bonds between the nitrogen atom of an amide and oxygen atom of a carbonyl group in secondary amide. Hydrogen bonds may form intra or inter chain of peptides, which result in the stability of gluten. Until now, there is no exact method to calculate the number of hydrogen bonds in gluten protein. Thus, this research, is one of the first attempt to present a unique method of obtaining the extent of hydrogen bonds in gluten. Hence, we selected two wheat cultivars, Morvarid and Sirvan. Hydrogen bonds were determined by FT-Raman spectroscopy and Scanning Thermal Analysis (STA). Results of FT-Raman Spectroscopy did not indicate the exact number of hydrogen bonds, However, it can qualitatively demonstrate the amount of hydrogen bonds. While the findings of STA analysis determined hydrogenbond of wheat gluten, both quantitatively and qualitatively. Thus, it is possible to evaluate the number of hydrogen bonds as one of the effective factors on gluten network stability, which can be correlated to wheat cultivars quality. In this work, we have shown that Sirvan cultivar has more hydrophilic amino acids as well as more hydrogen bonds than Morvarid cultivar.

Keywords: hydrogen bond; gluten; STA; FT-Raman spectroscopy Chemical compounds

Introduction

The aggregation of any particular protein involves a combination of both intramolecular and intermolecular interactions: the relative proportions of each type of interaction determine the overall folding and stability of the aggregated protein. Gluten isthe major protein of wheat. [1]. Technological properties of wheat is related to gluten network [1]. Gluten is composed of monomeric gliadin and polymeric glutenin fractions which is linked to each other by a covalent (disulfide) and noncovalent (hydrogen, ionic and hydrophobic) bonds [2]. The stability of primary structure of gluten is significantly attributed to interchain disulfide bonds. But folding and secondary structure of gluten are ascribed to hydrogen bond among peptides [3], which is formed between the oxygen atoms of the amide carbonyls (C=O) and nitrogen atoms (NH) of the secondary amides. There are two main types of secondary structure, the α-helices and the β-strand. These structures are defined by patterns of hydrogen bonds between the main-chain peptide groups. α-helices are present at the N- and C-terminals, while β- turns and β-sheets are present in the repetitive domain [4]. Due to the crucial role of special network formed upon hydration in bread making, the water sorption capability and water mobility in gluten dough is of great importance. The water absorption capacityand water mobility of flour products depend greatly on the distribution of polar groups, accessibility of these groups to water, relative strength of water-water and water- macromolecule interaction [5]. To determine the disulfide bonds, Ellman's method was mainly used [6], whereas to evaluate the quality and quantity of hydrogen bond, there is no exact method in gluten network. However, in FT-Raman spectroscopy, the tryptophan bonds at 880 cm^{-1} were used as an indicator of the strength of hydrogen bonding and $I_{850/830}$ was a monitor of the hydrogen bonding of the phenolic hydroxyl group in tyrosine [7, 8]. In this method, calculation of hydrogen bond is on the basis of tyrosine amino acid, while other amino acids containing hydroxyl groups can form hydrogen bond, so, FT-Raman is not an accurate method. Raman spectroscopy compared to FT-IR is not susceptible to interference from permanent dipole substances such as water, thus it is appropriate for analysis of proteins, DNA and RNA. In addition, Raman spectroscopy is opt for analyzing of polymer backbone structure, chain length, ring opening and conformation as well as intra and inter molecular vibrations [9]. DFT is a computational quantum mechanical modeling method used in physics, chemistry andmaterial science, to investigate the electronic structure (principally the ground state) [10] in particular atoms, molecules and the condensed phase. Using this theory, the properties of a many-electron system can be determined using functions, which in thiscase are the spatially dependent electron density. Hence, the name density functionaltheory comes from the use of functions of the electron density. Despite recent improvements, there are still difficulties in using density functional theory to properly describe intermolecular interactions. In addition, DFT method was used to determine hydrogen bond in identified structures, while DFT is not able to define unidentified differences between gluten networks of several cultivars. More importantly, simultaneous thermal analysis (STA) referes generally to the employing differential scanning calorimetry (DSC) and thermogravimetric analysis (TG) for the same sample at the same time [11], therefore STA is suitable for much low amount of samples, and is not time-consuming.

Bikadi, *et al*., 2007, used a network of hydrogen bonding interactions (HB plot) as a tool for exploring protein structure and function of protein. HB plot is obtained by plottingthe amino acid residues involved in hydrogen bonding horizontally and vertically [12].

Ferrer, *et al.* (2011), evaluated structural changes in gluten protein after the additionof sodium stearoyl lactylate (SSL) and the extent of modifications (secondary and tertiary structures) of this protein was analyzed using Raman spectroscopy [13].

Wang *et al*., 2014, investigated the effect of frozen storage on secondary structureand thermal properties of gluten, glutenin and gliadin-rich fractions by FTIR, TGA and DSC. Frozen storage induced reduction of α-helices structure and its conversion to specific β-sheet and β-turn structure and increasing denaturation temperature [14].

Shiraga *et al.,* 2016, investigated hydrogen bond network of water on a model protein (albumin), by the broadband complex dielectric constraint from 0.25 to 400 HZand IR-spectroscopy. Due to the topological and energetic disorder, the protein-water and water-water hydrogen bonding in the protein hydration shell are structurally incompatible with the native hydrogen bond network of bulk water. Hydration water around the protein is associated with the distorted hydrogen bond structure of hydrationwater, however, this distorted hydrogen bond network is not fragile, conversely, the hydrogen bond strength is greater and the population of fragmented nonhydrogen- bonded water is smaller for hydration water compared with that of bulk water [15].

Andrusenko *et al.,* (2016), evaluated three-dimensional hydrogen network in the structures of cobalt (II) complexes by DFT calculations and these interactions can beclassified as weak and moderate strength hydrogen bonds [16].

Mielcarek & Dolega (2016), studied weak hydrogen bonding interaction between the thiolate group of tri-tert-butoxysilanethiol (TBST) and carbonyl group of acetone by FT-IR spectroscopy. In order to confirm the interpretation of spectral results of hydrogenbonded system, DFT calculations have been used [17].

Evaluation effect of dietary fiber polysaccharides on structure and thermal propertiesof gluten proteins with application of FT-Raman spectroscopy, TGA and DSC in 2017, by Nawrocka *et al.,* indicated that these fibers induced changes in the secondary structure of gluten proteins concerning formation of antiparallel-β-sheets connected by intermolecular hydrogen bonds from α-helices and β-sheets [18].

As was observed in various research papers, there is no specific method to determine the quantity of hydrogen bonding in gluten. In most studies, secondarystructure changes of gluten,

which were created by exchanges of hydrogen bonding, were evaluated. Therefore,in this research, it is intended to represent an accurate method to determine content and strength of hydrogen bond in gluten network.

Materials and Methods

Materials

Two wheat cultivars (Morvarid and Sirvan) were selected from North andCentral parts of Iran, respectively.

All solvents/chemicals used were of analytical grade and obtained from Merck®(Germany).

Analysis

Chemical Analysis: Moisture, protein, wet gluten, falling number, Zeleny sedimentation value, weredetermined according to the methods established by the American Association ofCereal Chemists (2003) [19].

Amino acid analyzer: Hydrolysis was performed by mixing 10 mL HCl (6 M) with 0.1 g wheat flour for 20- 24 h at 110 °C. The mixture was held in a freezer (-18 °C) for an hour. The oxygen of the mixture was removed by blowing nitrogen gas and heated in the oven (110 °C) for 24 hours. The resulting solution was filtered and washed by deionized water three times. The solvent was separated by rotary evaporator in 60 °C, then added 2 mL amino acid analyzer buffer. Amino acid analysis was carried out by the *Spackman, et al.,* (1985) method, with amino acid analyzer (ARACUS). The chromatographic conditions are mobile phase, lithium citrate buffer, buffer flow rate, 220 µL/min, temperature range, 41-70 °C, analysis time, 90 min, 0.5 µM/mL amino acid mixture, [20]. The identification of amino acids were carried out during the retention time of amino acids. After the chromatographic separation of amino acid mixture, they react quantitatively with ninhydrin at 100-130 °C. Ninhydrin reacts with the amino group of amino acids, the resulting dimer has a blue dye. The intensity of the color is proportionalto the concentration of the dye and thus to the concentration of amino acid. Absorption was recorded photometrically at 570 nm and 440 nm. With proline and hydroxyproline, aslightly different reaction takes place which resulting dye has maximum absorption at 440 nm, whereas maximum absorption of other amino acids would be at 570 nm. The difference is attributed to reaction of ninhydrine with secondary amino groups (imino) of prolyne/hydroxyproline, as opposed to primary amino groups in others [21].

Extraction of Gluten: Wheat was milled by hammer milling (Perten, 3100), then was mixed with washing buffer (4.8 mL). Washing buffer included: sodium chloride (300 g), potassium dihydrogen phosphate (6.90 g) and disodium hydrogen phosphate (8.1g) in distilled water (15 lit). This suspension was washed with washing buffer using glutenwashing machine for 5 min until starch and bran were removed. The residue is viscoelastic gluten mass [19].

FT-Raman Spectroscopy: FT-Raman Spectroscopy was conducted in triplicate for each sample to determine the hydrogen bond of gluten proteins according to Ferrer *et al.,* (2011) [22]. Spectra values were obtained by Senterra Spectroscopy (Bruker, Germany), at room temperature, laser power of 100 mv, and resolution between 3-5 cm^{-1}. Each spectrum was obtained after a cummulative average of 1000 scans. FT-Raman spectra were plotted as a chart of intensity (arbitrary units) against Raman shift in wave number units (cm^{-1}). In Amide I region, a straight

baseline passing at 1700 and 1600 cm^{-1} in order to calculate this band intensity. The tryptophan bond at 880 cm^{-1} was used as an indicator of the strength of hydrogen bonding [23] and $I_{850/830}$ as a monitor of the hydrogen bonding. A decrease in $I_{850/830}$ ratio has been reported to reflect an increase inburiedness, suggesting the possible involvement of tyrosyl residues in intermolecular or intramolecular interaction. [13].

Denaturation of Hydrogen Bond in Gluten: Hydrogen bond was cleaved using a modified version of the procedure described by Gomez-Guillen, Borderias and Montero (1977) [24]. This approach is based on texture protein resolubilization by selective reagent that disrupt hydrogen bonds. Selective buffer prepared with urea (1.5 M) in phosphate buffer (0.05 M), pH=7.

STA Analysis: Simultaneous thermal analysis (STA) is a calorimetry analysis system, used to determine the mass and phase changes of material. Loss of water result in decrease in mass. Thermal treatment leads to the deformation of material. The required sample in STA instrument (BAHR 503) is in the range of 1mg to1g, analysis temperature is 0-1500 °C and rate of change in temperature is 1-100 °K/min. The reference sample is Al_2O_3. Atfirst, washed gluten network was floated in water for 24 h until the water is fully absorbed and then was subjected to air for 15 min to lose surface water. Prepared sample was transferred to STA. Absorbed water is attached to the surface of gluten via hydrogen bonds. On the other hand, some amino acids in gluten structure contribute to hydrogen bond, forming an interchain linkage between peptides. Therefore, amino acids which can absorb water are free, as a result, the more the amino acids are linked by hydrogen bonds, the least the water absorption.

To calculate the number of hydrogen bonds, lost mass difference between natural (ng) and denaturated (dg) gluten were determined by TGA. The mass difference(Δm) was related to hydrogen bonds in gluten network. Because one mole of any type of substance is equal to $6.02x10^{23}$ molecules of that specific substance; the count of hydrogen bonds are obtained based on the number of moles. The strength of hydrogen bonds was obtained from the differential thermal analysis (DTA). The area under of DTA peak is the enthalpy change of sample. As the minimum point in DTA graph is the beginning temperature of cleavage of hydrogen bonds, the higher the beginning temperature, the stronger the hydrogen bond.

Statistical Analysis: The statistical significance of the differences among wheat cultivars were determined by a one-way analysis of variance (ANOVA) using SPSS software. Duncananalysis was performed to differentiate and rank parameters using SPSS software, version 24. Differences were judged to be significant at $p < 0.05$.

Results and Discussion

Chemical and physicochemical analysis

Obviously, according to Table 1, quality and quantity of gluten in Sirvan cultivar ishigher than Morvarid cultivar. Consequently, Sirvan cultivar has stronger gluten network, because it has more linkages between gliadin and glutenin fractions.

FT-Raman Spectroscopy

Raman spectra of samples are shown in Figure 1. The tyrosine residues occur periodically throughout the length of gluten proteins and are involved in the gluten network formation during dough mixing by hydrogen bonding [25]. $I_{850/830}$ is 1.10 and 1.05 for Morvarid and Sirvan cultivars, respectively. Strength of hydrogen bond in Morvarid and Sirvan cultivars are 194.71 and 197.52 ev, respectively. Therefore, quantity and strength of hydrogen bond in Sirvan is greater than Morvarid cultivar. However, by this method, there is no significant difference between samples. On the other hand, FT-Raman Spectroscopy determined hydroxyl group of tyrosine, only [7], as wheat gluten has other amino acids containing hydroxyl group which are contributory factors in hydrogen bond formation. Also, the results of FT-Raman, which are used to determine hydrogen bonds, are not quantitative, exactly but, are comparative.

Table 1: Chemical and physicochemical characteristics of Morvarid and Sirvancultivars

Characteristics	Sirvan	Morvarid
Moisture (%)	$8.67^{a}\pm0$	$8^{b}\pm0.05$
Protein (%)	$12.65^{b}\pm0.4$	$13.21^{a}\pm0.01$
Wet gluten (%)	$31.35^{a}\pm0.0$	$25.0^{b}\pm0.50$
Sedimentation Zeleny value (ml)	$24.55^{a}\pm0.0$	$21^{b}\pm0.5$
Falling number (S)	$600^{a}\pm5$	$503^{b}\pm15$

Values followed by a different letter in the same row are significantly different ($P < 0.05$).

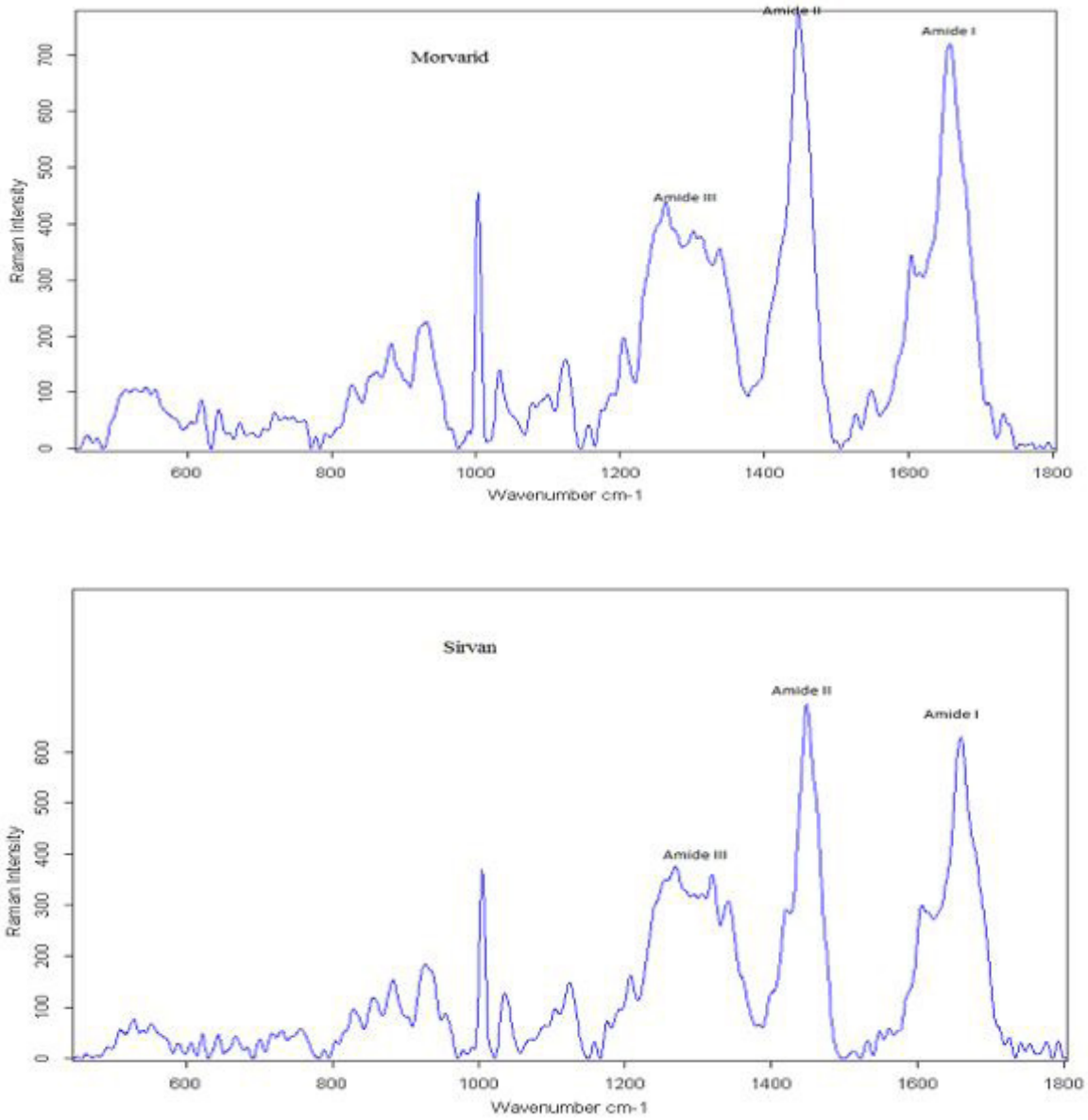


Figure 1: Raman spectra of gluten in Morvarid and Sirvan cultivars

Simultaneous Thermal Analysis (STA)

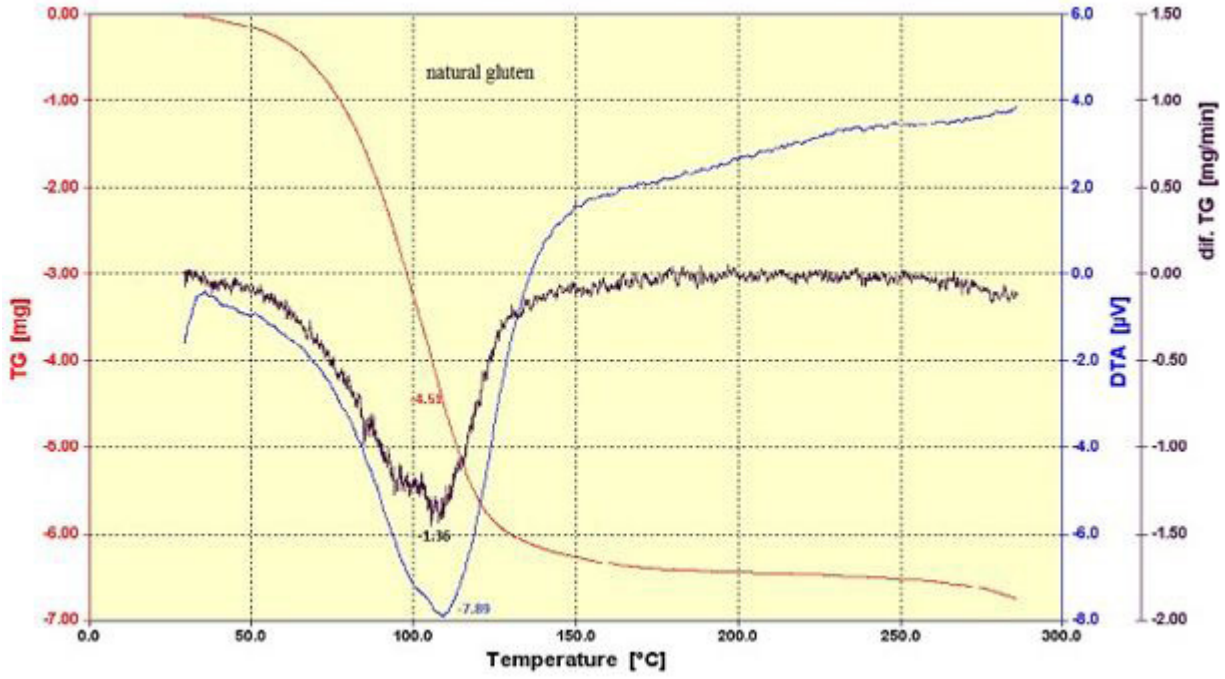
Generally, TGA profiles of gluten proteins could be divided into two steps, which were in accordance with the results of Khatkar *et al*., (2013) and Mohamed *et al*., (2008)[26, 27]. Initial weight loss in samples at 100-150 °C was due to the free and bonded water loss with the increasing temperature. Further degradation at higher temperatures are mainly involved with breakage of the covalent peptide bonds in the amino acid residues from protein molecules resulting in the decomposition of proteins [28, 29].

Wheat protein denaturation is one of major importance in establishing the bread structure [30]. Therefore, the alteration of gluten denaturation behavior may affect the final properties of bread. The surface-active properties of gluten have the potential to stabilize gas cells during bread making [31]. Falcao-Rodrigues (2005), proposed that the denaturation of gluten proteins is accompanied by decreased solubility and proceeds to a point where the gas cell walls are fixed and expansion ends. Hence, lower denaturation temperature of gluten would result in smaller gas cells and lower volume in bread. Figures 2 and 3 show mass and phase changes in gluten of Morvarid andSirvan.

On the basis of graphs, content of lost water in natural and denaturated gluten ofMorvarid and Sirvan cultivars were represented in Table 2. Therefore, in denaturated gluten, water absorption percent is greater than naturalgluten, because hydroxyl groups which contributed to the hydrogen bond between peptide chains, break down with urea and make them free, leading to the creation of new hydrogen bonds with water. Mass difference between denaturated and natural gluten corresponds to the interchain hydrogen bonds of peptides.

Table 2: Mass changes of gluten in Morvarid and Sirvan cultivars by TGA

Δm gluten (mg)	Morvarid	Sirvan
(Δng) natural gluten	0.574	0.629
(Δdg) denaturated gluten	0.594	0.703
(Δng) - (Δdg)	0.020	0.074



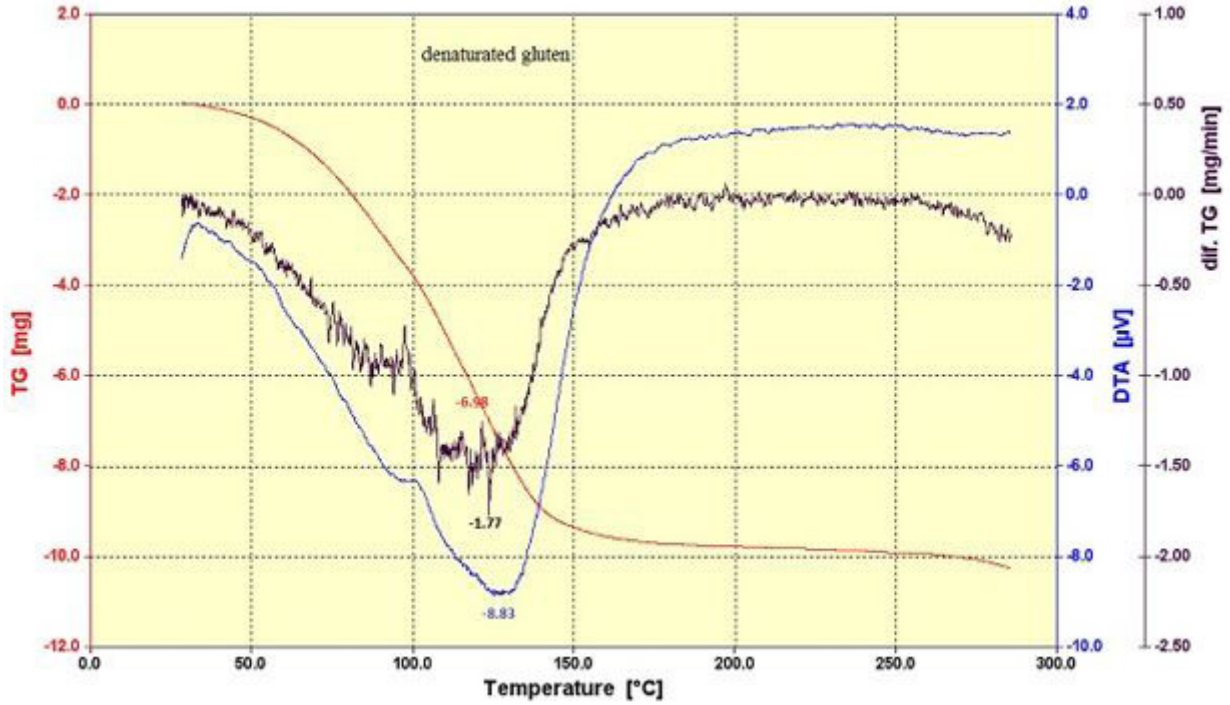
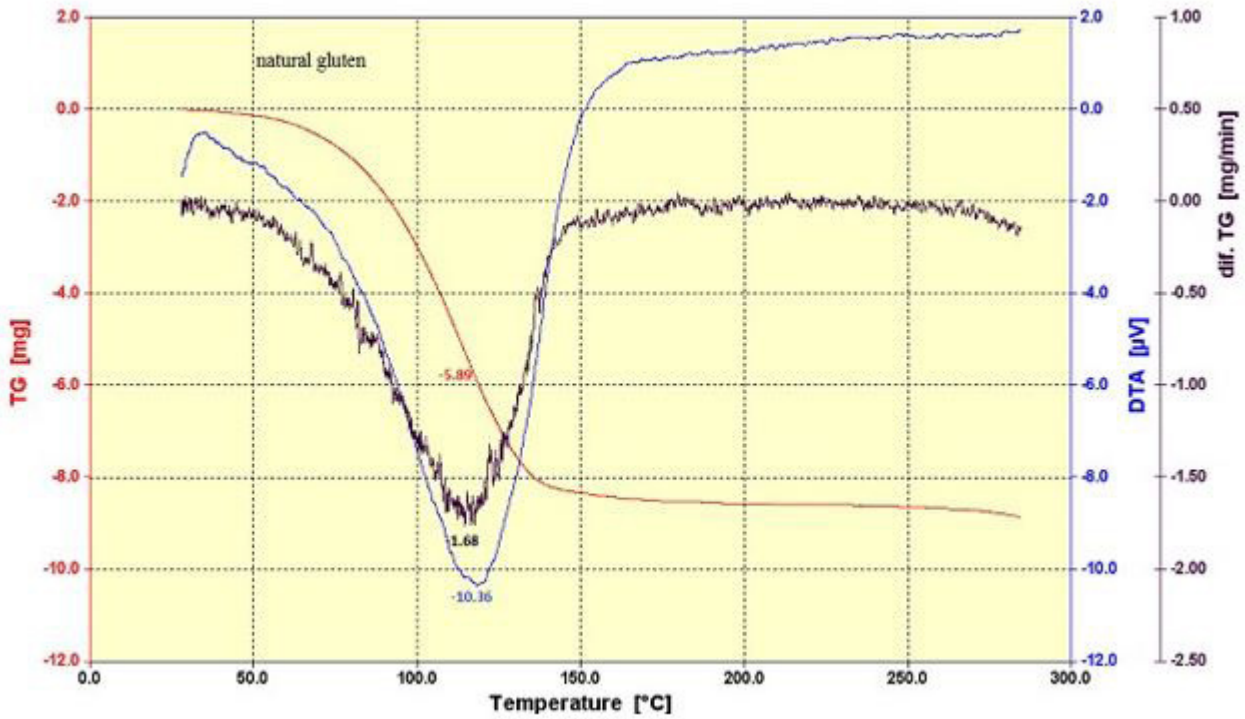


Figure 2: STA graph of gluten in Morvarid



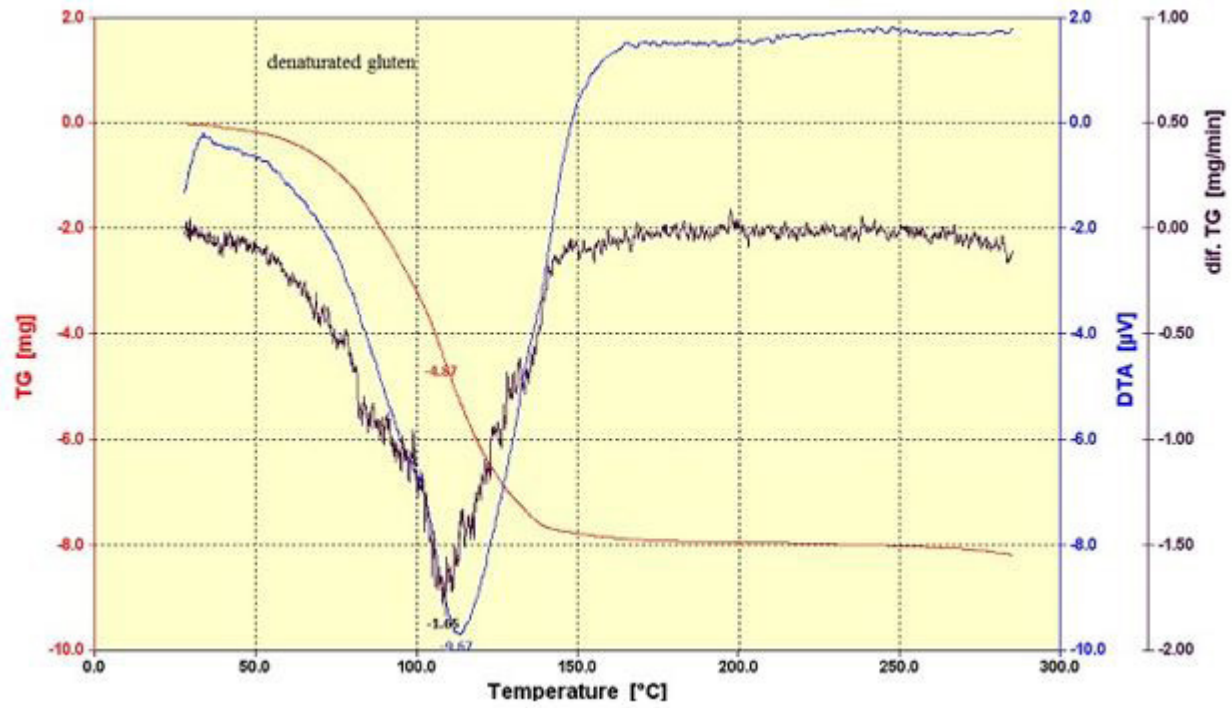


Figure 3: STA graph of gluten in Sirvan

Therefore the numberof hydrogen bonds in Morvarid and Sirvan cultivars are $6.7x10^{17}/2$ and $25x10^{17}/2$, respectively, because two hydroxyl groups form an hydrogen bond. Required temperature to break down hydrogen bonds or the denaturation temperature of natural gluten is 109.5 and 110.81 °C, in Morvarid and Sirvan cultivars, respectively. Therefore,quantity and strength of hydrogen bonds in Sirvan cultivar are higher than Morvarid cultivar.

Identification and quantification of hydrophilic and hydrophobic amino acids ofgluten

Chromatogram of Morvarid and Sirvan cultivars are depicted in Figure 4, that eachamino acid was identified from its retention time and its concentration was determinedfrom color intensity reaction with ninhydrin.

Sum of concentration of hydrophilic amino acids in Morvarid and Sirvan cultivars are 198.63 mg/Ll and 217.17 mg/L, respectively. In addition, concentration of hydrophobic amino acids in Morvarid and Sirvan cultivars are 58.02 mg/L and 57.09 mg/L, respectively. Because the content of hydrophilic amino acids in Sirvan cultivar is higherthan Morvarid cultivar, it is obvious that the extent of hydrogen bonds in Sirvan cultivar is greater than Morvarid, and this matter was confirmed by STA and FT-Raman spectroscopy.

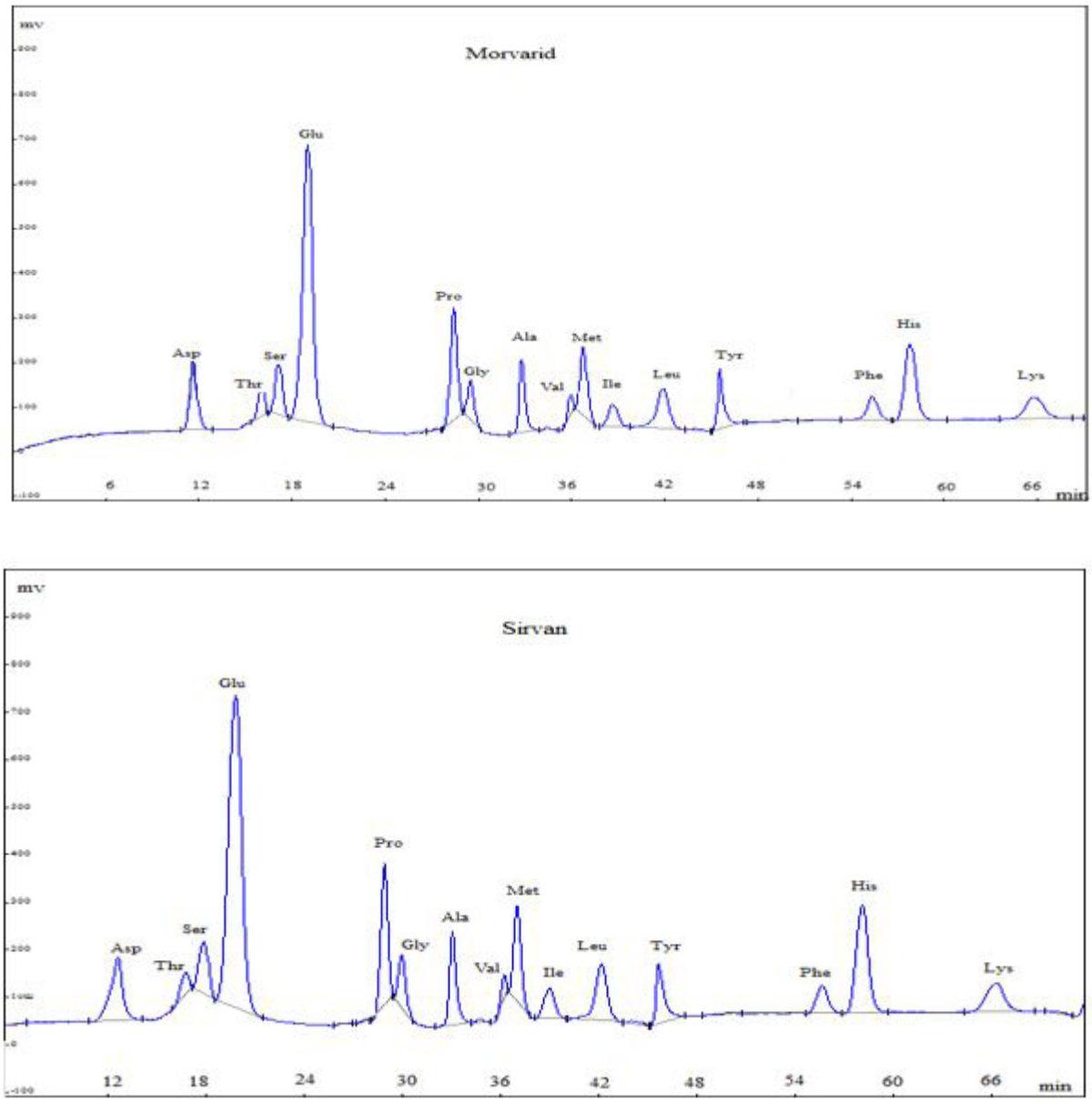


Figure 4: chromatogram of amino acid analyzer in Morvarid and Sirvan cultivars

Conclusion

Although hydrogen bonds' energies may seem small in the order of 4 to 25 KJ/mol [32], it should be kept in mind that they normally exist as an interconnecting network. As a result, they contribute greatly to the overall stability of the systems such as the polypeptide polymer. Intermolecular disulfide bonds in gluten network are of significant importance for building a gluten network [25, 33], whereas hydrogen bondsare much weaker than covalent bonds in gluten network [34]. On the other hand, the high levels of glutamine in the structure of wheat peptides is prone to form hydrogen bonds, along with wheat proteins are rich of glutamine. By this the influence of hydrogenbond in generating gluten network would be mediocre magnitude. Current methods of determining count of hydrogen bonds, is the use of DFT and FT-Raman spectroscopy.

While DFT method is a computational method is required to be obtained the structural model of samples. Therefore, this method is not applicable for detecting hydrogen bondin gluten network of several wheat cultivars in which preparation of structural models is extremely complicated.

Also, in FT-Raman Spectroscopy, the determination of hydrogen bond is on the basis of one amino acid, named tyrosine, but other amino acids containing hydroxyl group contribute to hydrogen bond formation. Therefore, up to now, there is

no other method to determine quantity of hydrogen bond, but STA analysis along with one treatment on gluten structure, is an effective method to calculate quantity and quality ofhydrogen bond.

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Conflict of Interest

This research did not receive any specific grants from any funding agencies inthe public, commercial, or not-for-profit sectors.

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