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# Study of structural changes of gluten proteins during bread dough mixing by Raman spectroscopy

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## Abstract

The aim of the present study was to evaluate Raman spectroscopy in determining changes that occur in the structure of gluten proteins induced during bread dough mixing. Raman spectra were measured directly within the dough. Three particular phases of mixing were studied: under-mixing, optimum mixing and over-mixing. A thiol blocking reagent, Tris(2-carboxyethyl)phosphine (TCEP) was then used to reduce disulphide bonds within proteins to confirm the important role of disulphide bridges in gluten network formation. For the control dough, the most important changes occurred during the optimum mixing phase when an increase in intermolecular disulphide bonds, anti-parallel  $\beta$ -sheet and  $\alpha$ -helix structures was observed, combined with the hydrophobic burial of tryptophan and tyrosine residues. The addition of TCEP appeared to effectively reduce the formation of intermolecular disulphide bonds, anti-parallel  $\beta$ -sheet and  $\alpha$ -helix structures and lead to a more disordered secondary protein structure.

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## Highlights

- Raman spectroscopy was used to study gluten structure changes during mixing.
- Raman spectroscopy measurements were performed directly in the dough matrix.
- A thiol blocking reagent was used as a reducing agent to reduce disulphide bonds.
- Intermolecular disulphide bonds are decisive for the stability of the gluten network.
- Hydrophobic bonds are important in the structural evolution of dough during mixing.

## Keywords

- Raman spectroscopy
- Bread dough mixing
- Gluten protein conformation
- Disulphide bond conformation
- Thiol blocking agent

## Chemical Compounds

Tris(2-carboxyethyl)phosphine (TCEP) (PubChem CID: 119411)

## 1. Introduction

Mixing is often considered as one of the most important steps in bread-making (Wieser, 2012). Indeed, several physical, chemical and physicochemical modifications occur during

dough mixing; in particular, the hydration of gluten proteins, gliadins and glutenins results in the formation of a continuous viscoelastic network. This gluten network contributes to gas holding capacity during fermentation and baking (Bloksma, 1990). The dough becomes more and more cohesive during mixing; however, if mixing continues beyond the threshold of maximum dough development, the dough weakens, then collapses and becomes sticky due to the increased mobility of its constituents (Sadot et al., 2017; Schiraldi & Fessas, 2012), leading to the production of lower quality bread. Monitoring mixing is therefore of paramount importance. Although very useful and performed on-line, torque, power and consistency measurements are limited to the description of the evolutions of the physical properties of dough. They do not give direct access to the chemical modifications of dough during the mixing-structuring step (Aït Kaddour & Cuq, 2011; Chin et al., 2004). However, these chemical properties can be assessed through spectroscopic investigations. Numerous studies have shown that vibrational spectroscopies are commonly used to study the structure of gluten protein (Aït Kaddour et al., 2008; Bock & Damodaran, 2013; Ferrer et al., 2011; Linlaud et al., 2011; Nawrocka et al., 2015, 2016a, 2016b, 2017a, 2017b, 2020; Rumińska et al., 2020; Seabourn et al., 2008). They are rapid, non-destructive, precise and reproducible, and generally do not require sample preparation. In addition, they can be used for qualitative and quantitative analyses and acquire several types of information simultaneously from a single spectrum (Chalmers & Griffiths, 2002). Among the spectroscopic methods available, both infrared and Raman spectroscopies can determine changes in the secondary structure of proteins (analysis of the Amide I and III bands) (Pelton & McLean, 2000), but Raman spectroscopy also gives information on the peptide backbone, the geometry of disulphide bonds, and the environment of certain side chains such as those of tyrosine, tryptophan, and methionine (Tu, 1982). Indeed, the stability of the gluten network formed during mixing is attributable to non-covalent or hydrophobic bonds as well as inter and intra-molecular

disulphide bonds (Shewry et al., 2000). Despite all these advantages, Raman spectroscopy has rarely been used for bread dough analysis. Ferrer et al. studied the influence of an emulsifier on gluten protein conformation (Ferrer et al., 2011). In their protocol, gluten samples extracted from wheat flour dough were freeze dried, milled by hand with a pestle in a mortar and stored at 4 °C before Raman analysis. Similarly, Nawrocka et al. (2015, 2016a, 2016b, 2017a, 2017b, 2020) conducted several studies on the impact of fibre addition on gluten protein conformation. In contrast, the work of Huen et al. (2014) showed that Raman spectra can be measured directly within a frozen dough, revealing the spatial distribution of the single components including gluten.

The main goal of this study was to use Raman spectroscopy to analyse the structural changes in gluten proteins that occur at different stages of mixing directly in dough samples, in order to determine whether this technique can be used to monitor the kneading process. As the role of SH (free thiol)/SS (disulphide bonds) interchange during mixing is crucial but not fully described in the literature (Belton, 2012; Wieser, 2012), the effect of a reducing agent is also studied in this paper. Indeed, it has been shown that the latter weakens the gluten matrix and induces a reduction in dough viscosity and elasticity (Bloksma, 1990). Therefore, we hypothesized that dough gluten protein should have a different and much less stable conformation when adding a thiolic blocking reagent. Tris(2-carboxyethyl)phosphine (TCEP) was chosen because it is often used to replace Dithiothreitol (DTT) and  $\beta$ -mercaptoethanol, and does not give rise to odours. Moreover, it provides selective, complete, quantitative and quick reduction (less than 5 min). An important advantage is that it is also active in both alkaline and acidic conditions (unlike DTT). In addition, it is more resistant to air oxidation and more hydrophilic. Above all, this molecule has the advantage of not having any S-H bonds that could mislead the interpretation.

## 2. Materials and methods

### 2.1. *Description of raw materials*

A soft bread dough formulation was used for all the tests. The ingredients used in the recipe per 100g of dough were: 60 g wheat flour (Moulins Soufflet SA - France), 33 g tap water, 2.4 g vegetable oil, 3 g sugar, 1.1 g salt and 0.6 g improver. The initial temperature of the dough was regulated by setting the sum of room, flour and water temperature to 55°C. Tris(2-carboxyethyl)phosphine (TCEP) was obtained from Thermo Fisher Scientific (Waltham, USA) in 0.5 M liquid solution. It was added to 0.075% (V/V) of the water in the recipe.

### 2.2. *Dough sample preparation*

The mixing process was carried out in a spiral mixer (SPI 11 – VMI, Montaigu, France) and included 2 phases. First, a pre-mixing step was performed at 100 rpm for 180 s in order to uniformly disperse the raw materials. Then, during the second phase, mixing was performed at 170 rpm. Mixing time was set by monitoring the power needed to actuate the rotational tool (spiral tool). The optimum mixing time was set at the power peak designated “ $t_{\text{PEAK}}$ ”. The dough samples were collected at three mixing levels: just after the pre-mixing step, at the optimum mixing time or  $t_{\text{PEAK}}$  and at over-mixing (Figure 1). For each sampling, three pieces of dough (~ 2 g) were transferred on a specific microscope slide with a cylindrical notch.

### 2.3. *Measurement*

The Raman spectra were acquired on a Senterra Raman microscope (Bruker Optik, GmbH). The samples were placed directly under a x100LWD objective using a 785 nm laser

excitation. The maximum laser power at the sample was 40 mW. The spectra were recorded over the range of 400-1800  $\text{cm}^{-1}$  with 400 gr/mm grating, providing a spectral resolution of about 9  $\text{cm}^{-1}$ . The calibration is done automatically by the patented SureCal<sup>TM</sup> technology. This option recalibrates continuously the device and allows permanent sub-wavenumber precision. For each mixing level, spectra were measured with three accumulations of 20 s per point at twelve different sample positions. All the measurements were performed in three independent experiments. All the results of these replications were reported in terms of average and standard deviation.

#### *2.4. Data processing*

Raman spectra were plotted as intensity (Arbitrary unit) against Raman shift ( $\text{cm}^{-1}$ ). Plot processing and handling (normalisation, baseline, band fitting) were carried out using OPUS software (Bruker Optik, GmbH). Each individual spectrum was first corrected from the baseline with a concave elastic correction method developed within OPUS software. Then, a min-max normalisation was applied. Finally, the 36 spectra obtained for each mixing level were averaged. To determine changes in the conformation of disulphide bonds, a spectral subtraction of a starch reference spectrum was required to completely remove the intense starch band at 478  $\text{cm}^{-1}$  which masks the contribution of the S-S bands. The conformations of disulphide bridges (500–560  $\text{cm}^{-1}$ ), Amide I band (1625–1700  $\text{cm}^{-1}$ ), Amide III band (1200–1330  $\text{cm}^{-1}$ ), tyrosine doublet (I(850)/I(830)), and tryptophan doublet (I(1360)/I(1340)) were analysed using a deconvolution by fitting a mix of Gaussian and Lorentzian curves (Figure S1, Figure S2, Figure S3, Figure S4 and Figure S5 are provided in Supplementary Material). The band assignment of the main vibrations of the side chains and the secondary structure was based on comparison to Raman data reported in the literature, in particular for the disulphide bridge: interchain disulphide bonds gauche-gauche-gauche (g-g-g) around 500  $\text{cm}^{-1}$ ,

intrachain disulphide bonds trans-gauche-gauche (t-g-g) at 510-520  $\text{cm}^{-1}$  , trans-gauche-gauche (t-g-g) at 525-535  $\text{cm}^{-1}$ , and trans-gauche-trans (t-g-t) at 540-545  $\text{cm}^{-1}$  (Ferrer et al., 2011); for the Amide I band:  $\beta$ -sheets ( $\beta$ S) at 1630-1640  $\text{cm}^{-1}$ , Random Coil (RC) at 1640-1650  $\text{cm}^{-1}$  ,  $\alpha$ -helix ( $\alpha$ H) at 1650-1658  $\text{cm}^{-1}$ ,  $\beta$ -turns ( $\beta$ T) at 1670-1678  $\text{cm}^{-1}$  and antiparallel- $\beta$ -sheets ( $\alpha\beta$ S) at 1690-1700  $\text{cm}^{-1}$  and finally for the Amide III band:  $\beta$ -sheets ( $\beta$ S) at 1220-1250  $\text{cm}^{-1}$ , Random Coil (RC) at 1250-1270  $\text{cm}^{-1}$ ,  $\beta$ -turns ( $\beta$ T) at 1270-1295  $\text{cm}^{-1}$  , and  $\alpha$ -helix ( $\alpha$ H) at 1295-1330  $\text{cm}^{-1}$  (Nawrocka et al., 2020).

### 3. Results and Discussion

#### *3.1. Dough formation monitoring*

Dough development was monitored during mixing by the change in the power curve (Figure 1). At slow speed, the dough still had very low resistance, so the power consumed by the tool to maintain its speed was also very low. The energy consumed every second therefore increased only slightly. When the speed of the tool changed, the development phase of the protein network began. With the kneading time, the energy provided by the agitation was used for the formation of the gluten network, resulting in an increase in viscoelasticity and therefore resistance. The power consumption then increased during the development phase. After a certain kneading time, the power curve reached a maximum and then decreased. This maximum, corresponding to a maximum of viscoelasticity, was a remarkable point of the "t<sub>PEAK</sub>" kneading for the "time to reach the power peak" (Heaps et al., 1967). The t<sub>PEAK</sub> is very close to the optimal kneading time determined empirically by bakers (Sadot et al., 2017). Once t<sub>PEAK</sub> was reached, further kneading led to an overmixing phase, where the dough underwent a change in the state of hydration of the gluten network and in the distribution of water (Schiraldi & Fessas, 2012).



For the control dough, the development time was about 475s (Figure 1). As expected (Wieser, 2012), the addition of TCEP considerably reduced the dough development time (345s) (Figure 1). The maximum absorbed power was slightly higher than that of the control dough, meaning that either SH/SS interchange or other non-covalent interactions (hydrogen, ionic and hydrophobic bonds) remained sufficient to create a resistant gluten network, with probably a larger glutenin fraction. The faster decrease of the power suggested that this network was unstable and collapsed with the mechanical action of the mixing tool, rapidly leading to an over-mixing state. This could be due to excessive interchange (Wieser, 2012). From the experimental point of view, the dough with TCEP was effectively clearly destructured and very sticky at the end of the experiment compared to the control dough.

### *3.2. Changes in secondary structure (Amide I and Amide III)*

The secondary structure of proteins was studied by Raman spectroscopy using the Amide I (1600-1700  $\text{cm}^{-1}$ ) and Amide III (1200-1330  $\text{cm}^{-1}$ ) bands. The Amide I region obtained for control dough and the dough with the addition of TCEP at the three different mixing levels is shown in Supplementary Material (Figure S7). As expected in the literature (Ferrer et al., 2011), the  $\alpha$ -helix was the main dominant structure with an intense amide I band located at 1656  $\text{cm}^{-1}$ . An increase of the  $\alpha$ -helix content accompanied with a decrease in random coil and  $\beta$ -turn structures until the optimum mixing level was observed for the control dough (Figure 2). The bands related to the parallel- $\beta$ -sheet were constant during mixing while a large increase in the antiparallel- $\beta$ -sheet was observed at the optimum mixing level (Figure 2). These modifications are related to protein folding, suggesting conformational changes that lead to a more ordered structure. The  $\alpha$ -helix and antiparallel- $\beta$ -sheet contents were clearly less significant in the presence of TCEP although evolved to a lesser extent in the same way

as the control dough (Figure 2). However, the random coil and the  $\beta$ -turn structures tended to increase during mixing. A similar tendency was observed for the  $\alpha$ -helix,  $\beta$ -sheet, random coil and  $\beta$ -turn components in the Amide III band (Figure 3). The addition of TCEP seemed to diminish the formation of the antiparallel- $\beta$ -sheet and favour the presence of random coil and  $\beta$ -turn structures (Figure 2 and Figure 3).

### 3.3. Disulphide bonds

Among covalent and non-covalent bonds, disulphide bonds (SS) play a key role in dough structure, forming either intramolecular disulphide bonds within a protein or intermolecular disulphide bonds between protein chains. Generally in the literature, the attribution of disulphide bands is based almost exclusively on frequency values between 500 and 560 $\text{cm}^{-1}$ . The difference between the values of these frequencies are commonly attributed to different conformations of the dihedral angles  $C_{\alpha}-C_{\beta}-S-S'-C_{\beta}'-C_{\alpha}'$ . A vibration around  $510 \pm 5 \text{ cm}^{-1}$  can be assigned to the gauche-gauche-gauche (g-g-g) conformation, around  $525 \pm 5 \text{ cm}^{-1}$  to the gauche-trans-gauche (t-g-g) conformation and around  $540 \pm 5 \text{ cm}^{-1}$  to the trans-gauche-trans (t-g-t) conformation (Tu, 1982). The g-g-g conformation is considered more stable than the other two and a distinction can be made between intermolecular S-S linkage with a band localised around 497  $\text{cm}^{-1}$  and intramolecular S-S linkage with a band localised around 514  $\text{cm}^{-1}$  (Ferrer et al., 2011). A large increase in intermolecular S-S disulphide bonds was observed until the optimum mixing time followed by a significant decrease of both intermolecular and intramolecular S-S conformation for over-mixed dough (Figure 4). This observation is consistent with the “loop and train” model described by Belton (Belton, 2012), where an increase of the displacement implies a rupture of the trains and thus the breakage of

the intermolecular disulphide bonds. Other SS conformations remained quite stable during mixing (Figure 4).

The addition of TCEP seemed to prevent the formation of disulphide intermolecular S-S links, since the band around  $500\text{ cm}^{-1}$  stayed the same during mixing and, on the contrary, to favour the t-g-g conformation (Figure 4), which is considered less stable.

### *3.4. Environment of aromatic acid (tyrosine and tryptophan)*

Changes in the aromatic acid environment can also be detected by Raman spectroscopy, especially for two amino acids: tyrosine (TYR) and tryptophan (TRP). The ratio of the tyrosine doublet ( $830$  and  $850\text{ cm}^{-1}$ ) provides information on hydrogen bonding by the phenolic hydroxyl groups, whereas the ratio of the tryptophan doublet ( $1340$  and  $1360\text{ cm}^{-1}$ ) is considered to be a marker of hydrophobicity of the indole ring (Tu, 1982).

A ratio greater than one for the TYR I850/I830 doublet for under-mixed dough is reported in Figure 5. This means that the tyrosine residues were exposed and played the role of both acceptor or donor of a proton, thus favouring interaction with water molecules. Then, a significant decrease of the TYR I850/I830 ratio at the optimum mixing time was observed, reflecting an increase in buriedness that could result in the folding or aggregation of gluten proteins (Nawrocka et al., 2017a). However, as the value of the ratio was still close to one, it could still be treated as a ‘normal tyrosine’ that acts as both donor and acceptor of a proton but with a greater part of the TYR residues participating in the H-bonds as a proton donor (Rumińska et al., 2020). This observation is consistent with the fact that there is less water mobility at the optimum mixing time (Schiraldi & Fessas, 2012). Finally, a noticeable increase for over-mixed dough confirmed that the mobility of water was once again possible,

making the dough stickier (Schiraldi & Fessas, 2012). The same behaviour was observed with the addition of TCEP, which did not have any impact on the TYR residues (Figure 5).

The intensity ratio  $I_{1360}/I_{1340}$  is particularly sensitive to the transition of tryptophan microenvironments from a hydrophobic one to a hydrophilic one. An increase of the ratio indicates a buriedness of the tryptophan residues while a decrease indicates their exposure to a polar environment (Takeuchi, 2003). For under-mixed dough (Figure 6), the available water at the beginning of the mixing process can fill the pores of the gluten proteins, which provide a large solvent accessible surface. At optimum mixing time (Figure 6), the increase of the doublet ratio indicates an increase in the buriedness that suggests that the tryptophan residues contribute to a more ordered structure: less free water is available and bound water has multiple contacts that stabilize the structure of the protein. This increase may also support the hypothesis of partial dehydration of the gluten network during mixing (Nawrocka et al., 2017). When dough is overmixed (Figure 6), the release of water leads to the destruction of the hydrophobic cluster structure, as indicated by the sharp decrease in the doublet ratio. For over-mixed dough, the ratio decreased, meaning that the TRP residues returned to a polar environment. The same behaviour was observed for the dough with TCEP (Figure 6).

#### 4. Conclusion

To our knowledge, this study is the first attempt to describe the structural changes in gluten proteins induced by mixing and measured directly in the dough by Raman spectroscopy. The results obtained are promising because specific behaviours were observed at successive stages of mixing. This Raman Spectroscopy study confirmed that at the optimum mixing time, a clear

increase in intermolecular g-g-g disulphide bond conformation appeared, associated with an increase in the  $\alpha$ -helix and anti-parallel- $\beta$ -sheet structures, and with the hydrophobic burial of tryptophan and tyrosine residues. These changes imply a more ordered structure that favours the stability of the gluten network when optimal mixing is reached. Indeed, inter-chain SS bonds giving rise to stiffer polymer and  $\beta$ -spiral structures may contribute to gluten elasticity through both intrinsic elasticity and the formation of loops and trains stabilised by hydrogen bonds (Shewry et al., 2000). When dough was over-mixed, the gluten structure was somewhat disordered (decrease of intermolecular g-g-g disulphide bonds, increase of Random Coil conformation) and the gluten network weakened. The addition of a thiolic blocking reagent (TCEP) seemed to reduce the formation of SS intermolecular g-g-g conformation, the anti-parallel- $\beta$ -sheet and  $\alpha$ -helix structures, without modifying the hydrogen bonds. The dough could still develop, reaching maximum resistance that was obtained more rapidly than for the control dough. However, resistance to extension was very fragile and the quality of the resulting dough was clearly poor, with a lack of viscosity and elasticity. It therefore appeared that intermolecular disulphide bonds are decisive in the structural evolution of dough during mixing and for the stability of the gluten network. Further investigations are needed to determine whether changes in disulphide bridge conformation are the cause or effect of the changes in the secondary protein structure observed.

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## **Declaration of Competing Interest**

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

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Figure 1: Power mixing curves. Evolution of power during mixing, illustrating the effect of adding TCEP to a bread dough recipe.

Figure 2: Raman determination of Amide I band secondary structure for Control dough and Dough + TCEP for the three different mixing levels: Under-mixing, Optimum and Over-mixing ( $\beta$ S – parallel- $\beta$ -sheet, RC- Random Coil,  $\alpha$ H –  $\alpha$ -helix,  $\beta$ T – $\beta$ -turns,  $a\beta$ S – antiparallel- $\beta$ -sheet).

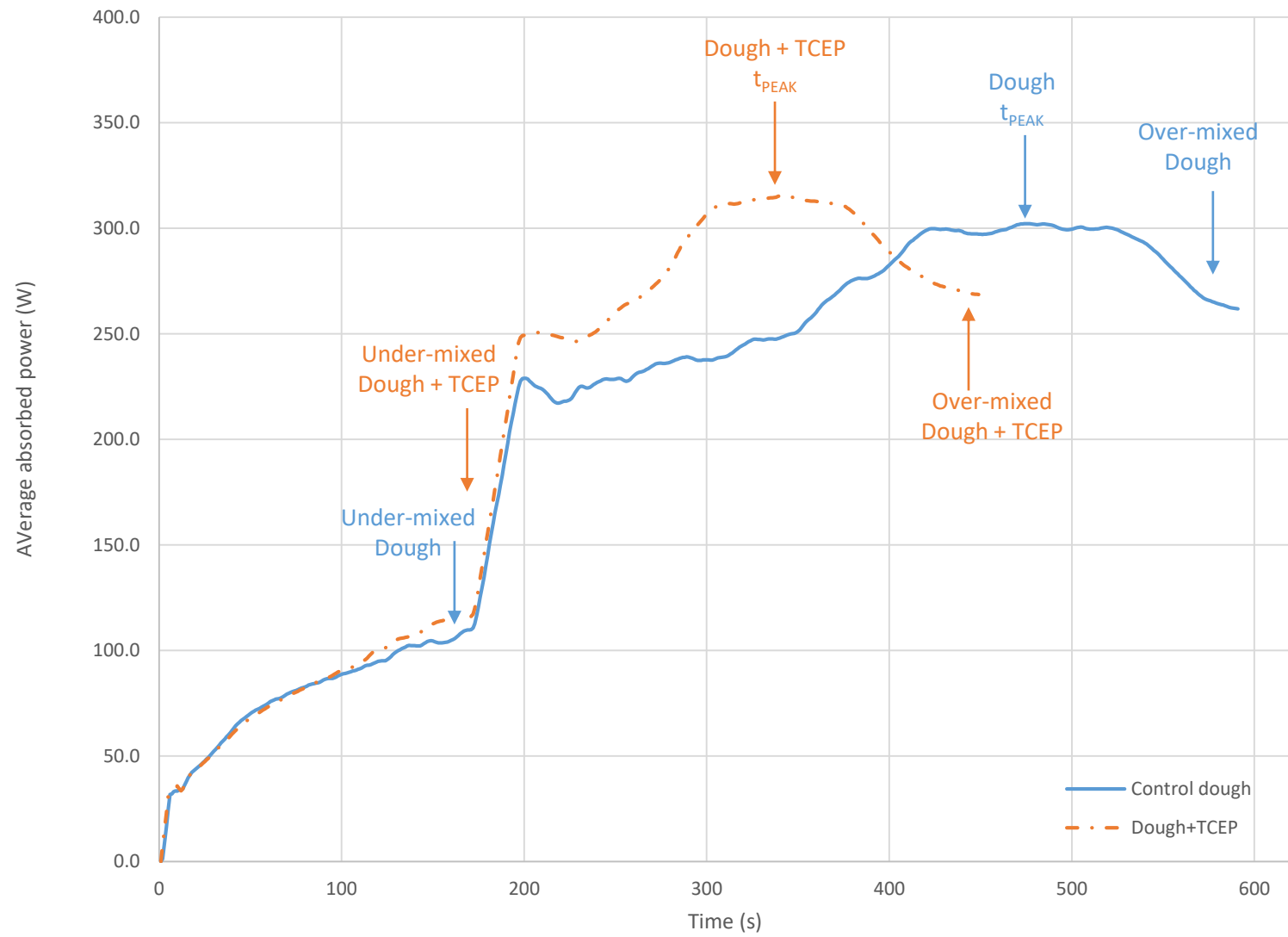
Figure 3: Raman determination of Amide III band secondary structure for Control dough and Dough + TCEP for the three different level of mixing Under-mixing, Optimum and Over-mixing ( $\beta$ S –  $\beta$ -sheet, RC- Random Coil,  $\beta$ T – $\beta$ -turns,  $\alpha$ H –  $\alpha$ -helix).

Figure 4: Raman determination of Disulphide bridge conformation for Control dough and Dough + TCEP for the three different mixing levels: Under-mixing, Optimum and Over-mixing (g-g-g (inter SS) conformation, t-g-g (intra SS) conformation, t-g-g conformation and t-g-t conformation).

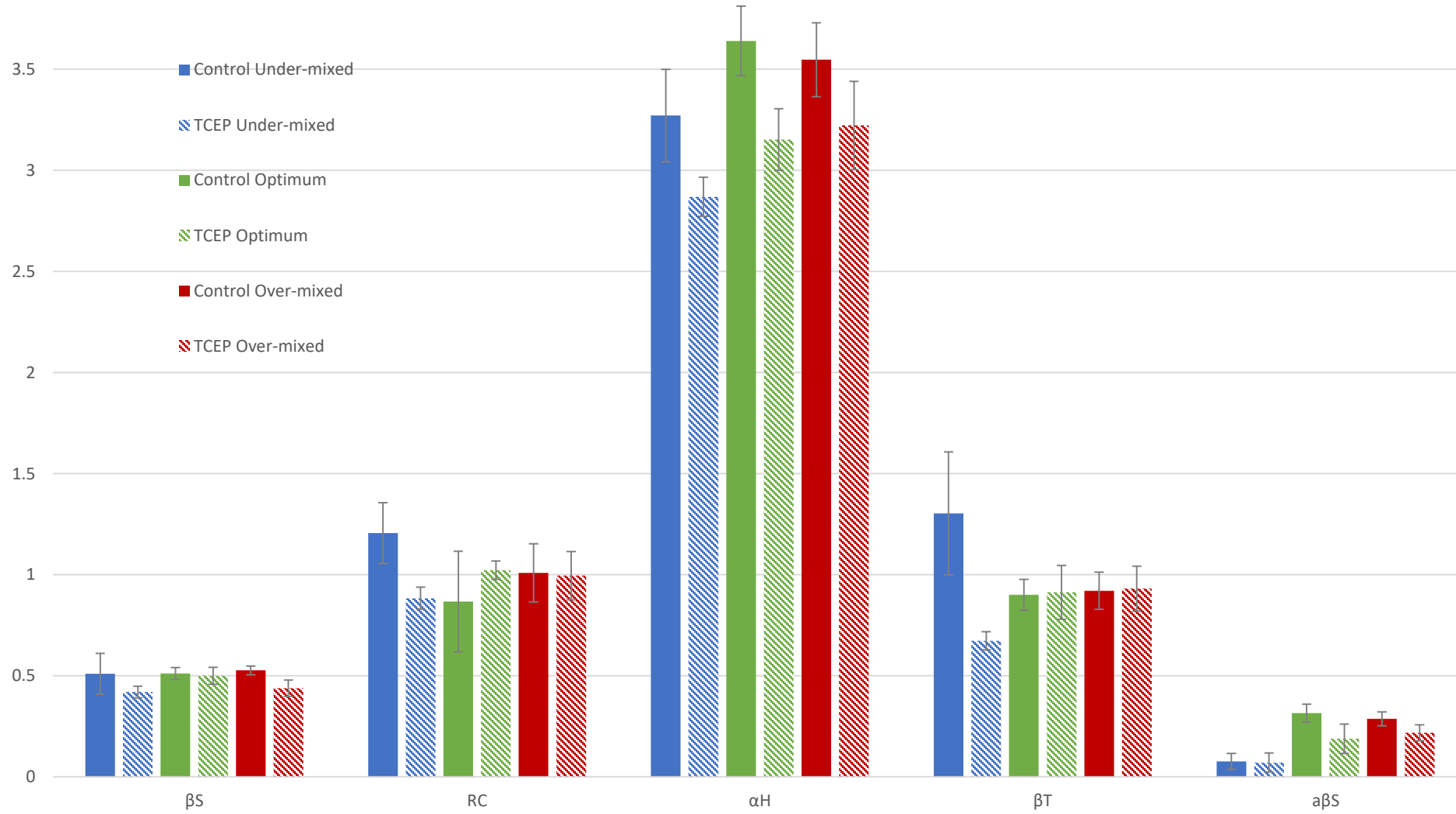
Figure 5: Raman determination of the Tyrosine doublet ratio for Control dough and Dough + TCEP for the three different mixing levels: Under-mixing, Optimum and Over-mixing.

Figure 6: Raman determination of the Tryptophan doublet ratio for Control dough and Dough + TCEP for the three different mixing levels: Under-mixing, Optimum and Over-mixing.

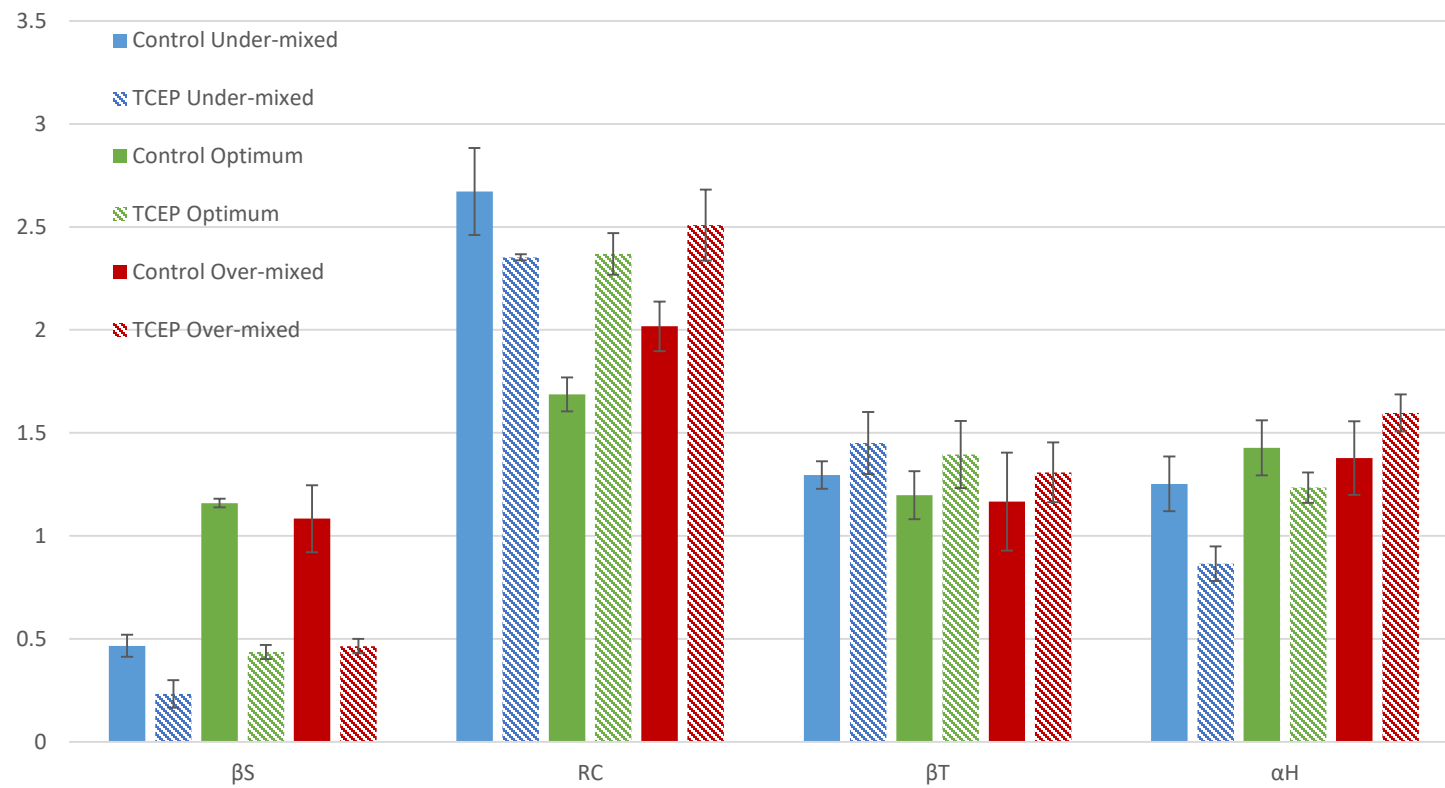




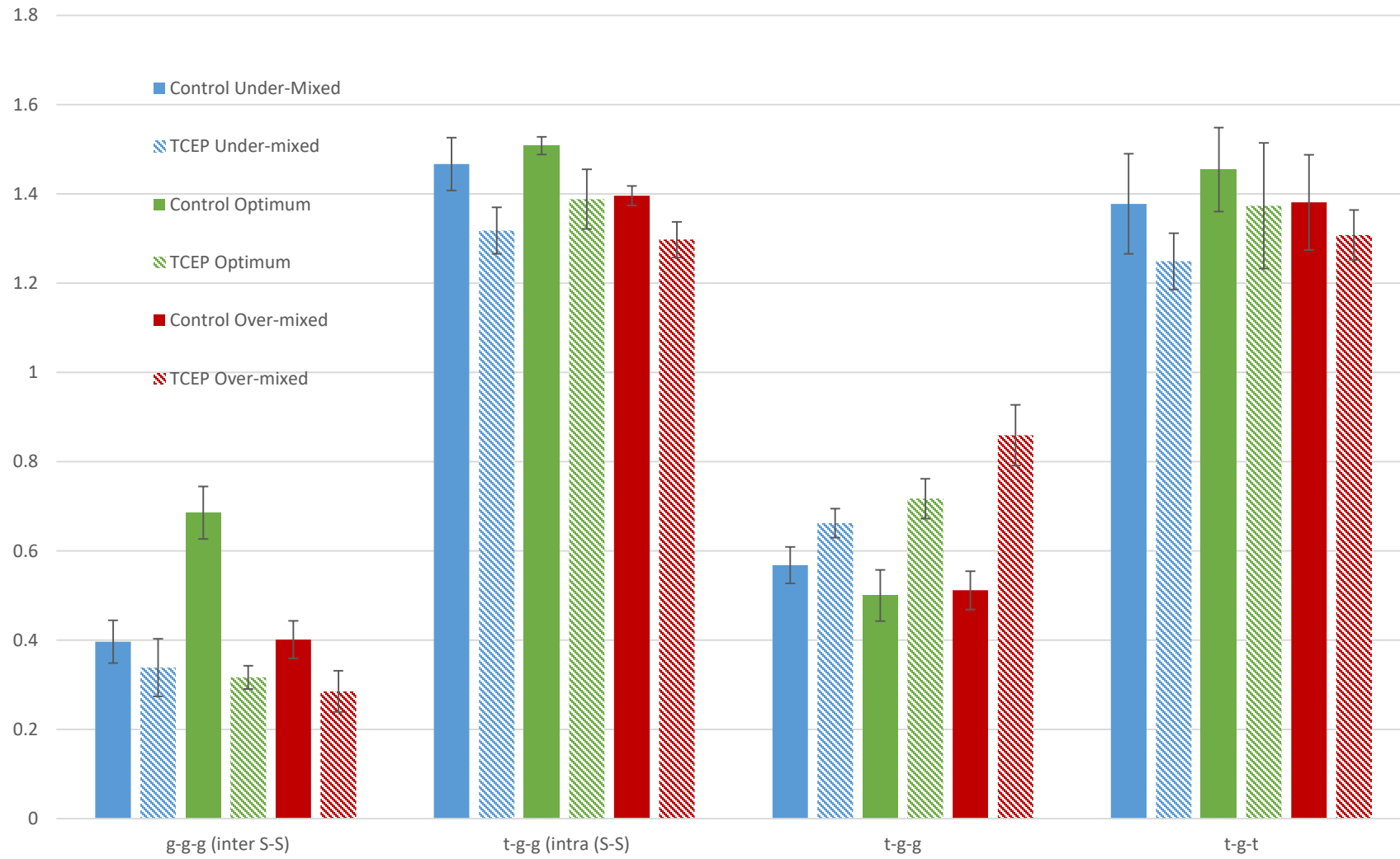
## Amide I



### Amide III



## Disulphide bonds



## Tyrosine

