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1 Study of structural changes of gluten proteins during bread dough mixing 2 by Raman spectroscopy

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8 Abstract

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

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22

23 **Highlights**

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

29

30 **Keywords**

- 31 • Raman spectroscopy
- 32 • Bread dough mixing
- 33 • Gluten protein conformation
- 34 • Disulphide bond conformation
- 35 • Thiol blocking agent

36

37 **Chemical Compounds**

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

39

40 **1. Introduction**

41 Mixing is often considered as one of the most important steps in bread-making (Wieser,
42 2012). Indeed, several physical, chemical and physicochemical modifications occur during

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

68 disulphide bonds (Shewry et al., 2000). Despite all these advantages, Raman spectroscopy has
69 rarely been used for bread dough analysis. Ferrer et al. studied the influence of an emulsifier
70 on gluten protein conformation (Ferrer et al., 2011). In their protocol, gluten samples
71 extracted from wheat flour dough were freeze dried, milled by hand with a pestle in a mortar
72 and stored at 4 °C before Raman analysis. Similarly, Nawrocka et al. (2015, 2016a, 2016b,
73 2017a, 2017b, 2020) conducted several studies on the impact of fibre addition on gluten
74 protein conformation. In contrast, the work of Huen et al. (2014) showed that Raman spectra
75 can be measured directly within a frozen dough, revealing the spatial distribution of the single
76 components including gluten.

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

93 2. Materials and methods

94

95 2.1. *Description of raw materials*

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

103 2.2. *Dough sample preparation*

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

112

113 2.3. *Measurement*

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

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

124 *2.4. Data processing*

125 Raman spectra were plotted as intensity (Arbitrary unit) against Raman shift (cm^{-1}). Plot
126 processing and handling (normalisation, baseline, band fitting) were carried out using OPUS
127 software (Bruker Optik, GmbH). Each individual spectrum was first corrected from the
128 baseline with a concave elastic correction method developed within OPUS software. Then, a
129 min-max normalisation was applied. Finally, the 36 spectra obtained for each mixing level
130 were averaged. To determine changes in the conformation of disulphide bonds, a spectral
131 subtraction of a starch reference spectrum was required to completely remove the intense
132 starch band at 478 cm^{-1} which masks the contribution of the S-S bands. The conformations of
133 disulphide bridges (500–560 cm^{-1}), Amide I band (1625–1700 cm^{-1}), Amide III band (1200-
134 1330 cm^{-1}), tyrosine doublet (I(850)/I(830)), and tryptophan doublet (I(1360)/I(1340)) were
135 analysed using a deconvolution by fitting a mix of Gaussian and Lorentzian curves (Figure
136 S1, Figure S2, Figure S3, Figure S4 and Figure S5 are provided in Supplementary Material).
137 The band assignment of the main vibrations of the side chains and the secondary structure was
138 based on comparison to Raman data reported in the literature, in particular for the disulphide
139 bridge: interchain disulphide bonds gauche-gauche-gauche (g-g-g) around 500 cm^{-1} ,

140 intrachain disulphide bonds trans-gauche-gauche (t-g-g) at 510-520 cm^{-1} , trans-gauche-
141 gauche (t-g-g) at 525-535 cm^{-1} , and trans-gauche-trans (t-g-t) at 540-545 cm^{-1} (Ferrer et al.,
142 2011); for the Amide I band: β -sheets (β S) at 1630-1640 cm^{-1} , Random Coil (RC) at 1640-
143 1650 cm^{-1} , α -helix (α H) at 1650-1658 cm^{-1} , β -turns (β T) at 1670-1678 cm^{-1} and antiparallel-
144 β -sheets ($\alpha\beta$ S) at 1690-1700 cm^{-1} and finally for the Amide III band: β -sheets (β S) at 1220-
145 1250 cm^{-1} , Random Coil (RC) at 1250-1270 cm^{-1} , β -turns (β T) at 1270-1295 cm^{-1} , and α -
146 helix (α H) at 1295-1330 cm^{-1} (Nawrocka et al., 2020).

147 3. Results and Discussion

148 3.1. Dough formation monitoring

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

163 For the control dough, the development time was about 475s (Figure 1). As expected
164 (Wieser, 2012), the addition of TCEP considerably reduced the dough development time
165 (345s) (Figure 1). The maximum absorbed power was slightly higher than that of the control
166 dough, meaning that either SH/SS interchange or other non-covalent interactions (hydrogen,
167 ionic and hydrophobic bonds) remained sufficient to create a resistant gluten network, with
168 probably a larger glutenin fraction. The faster decrease of the power suggested that this
169 network was unstable and collapsed with the mechanical action of the mixing tool, rapidly
170 leading to an over-mixing state. This could be due to excessive interchange (Wieser, 2012).
171 From the experimental point of view, the dough with TCEP was effectively clearly
172 destructured and very sticky at the end of the experiment compared to the control dough.

173

174 3.2. Changes in secondary structure (Amide I and Amide III)

175 The secondary structure of proteins was studied by Raman spectroscopy using the Amide
176 I (1600-1700 cm^{-1}) and Amide III (1200-1330 cm^{-1}) bands. The Amide I region obtained for
177 control dough and the dough with the addition of TCEP at the three different mixing levels is
178 shown in Supplementary Material (Figure S7). As expected in the literature (Ferrer et al.,
179 2011), the α -helix was the main dominant structure with an intense amide I band located at
180 1656 cm^{-1} . An increase of the α -helix content accompanied with a decrease in random coil
181 and β -turn structures until the optimum mixing level was observed for the control dough
182 (Figure 2). The bands related to the parallel- β -sheet were constant during mixing while a large
183 increase in the antiparallel- β -sheet was observed at the optimum mixing level (Figure 2).
184 These modifications are related to protein folding, suggesting conformational changes that
185 lead to a more ordered structure. The α -helix and antiparallel- β -sheet contents were clearly
186 less significant in the presence of TCEP although evolved to a lesser extent in the same way

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

192

193 *3.3. Disulphide bonds*

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

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

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

215

216 *3.4. Environment of aromatic acid (tyrosine and tryptophan)*

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

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

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

235

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

250

251 **4. Conclusion**

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

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

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277 **Declaration of Competing Interest**

278 The authors declare that they have no known competing financial interests or personal
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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.

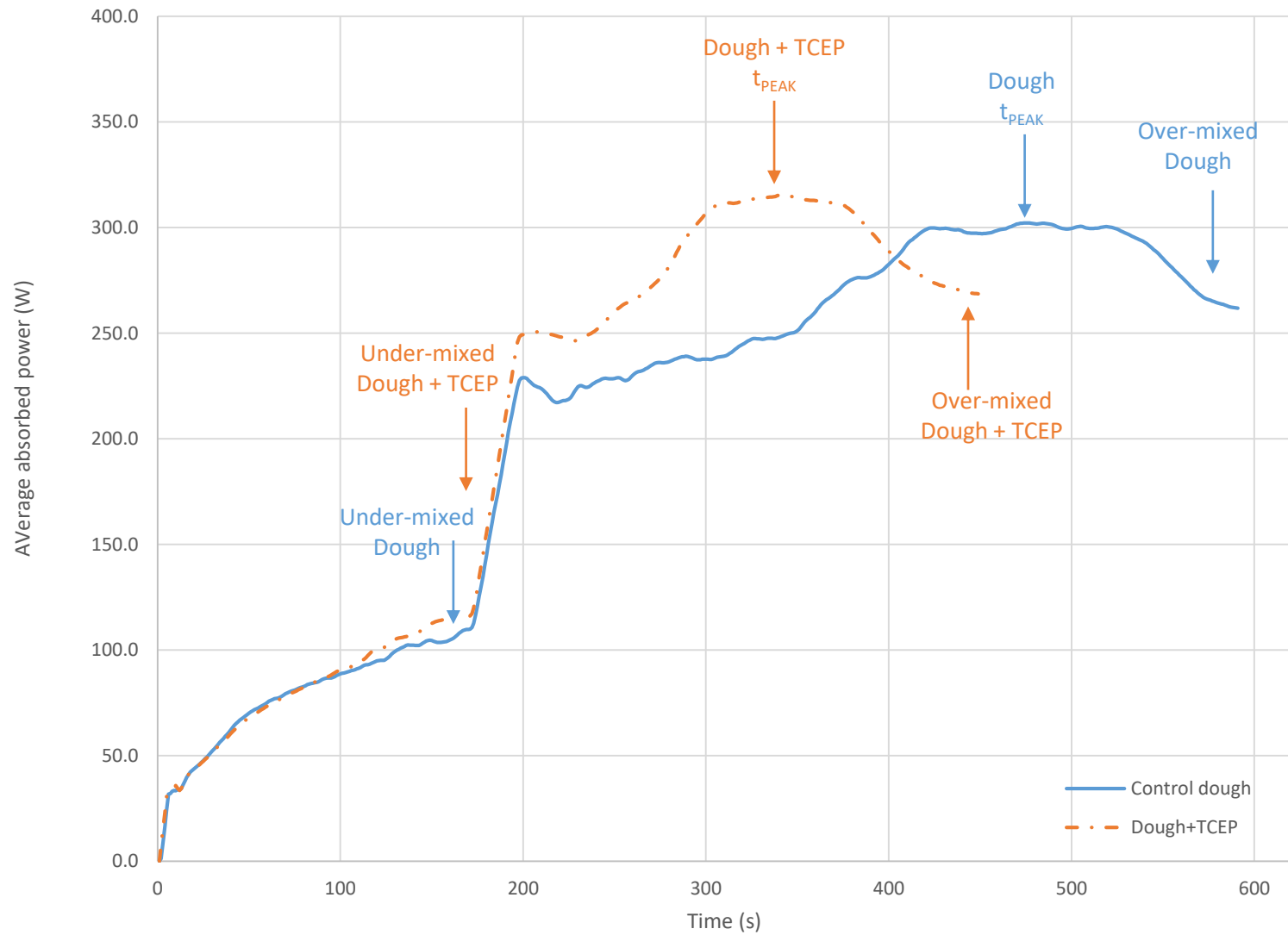
Figure 2: Raman determination of Amide I band secondary structure for Control dough and Dough + TCEP for the three different mixing levels: Under-mixing, Optimum and Over-mixing (β S – parallel- β -sheet, RC- Random Coil, α H – α -helix, β T – β -turns, $a\beta$ S – antiparallel- β -sheet).

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 (β S – β -sheet, RC- Random Coil, β T – β -turns, α H – α -helix).

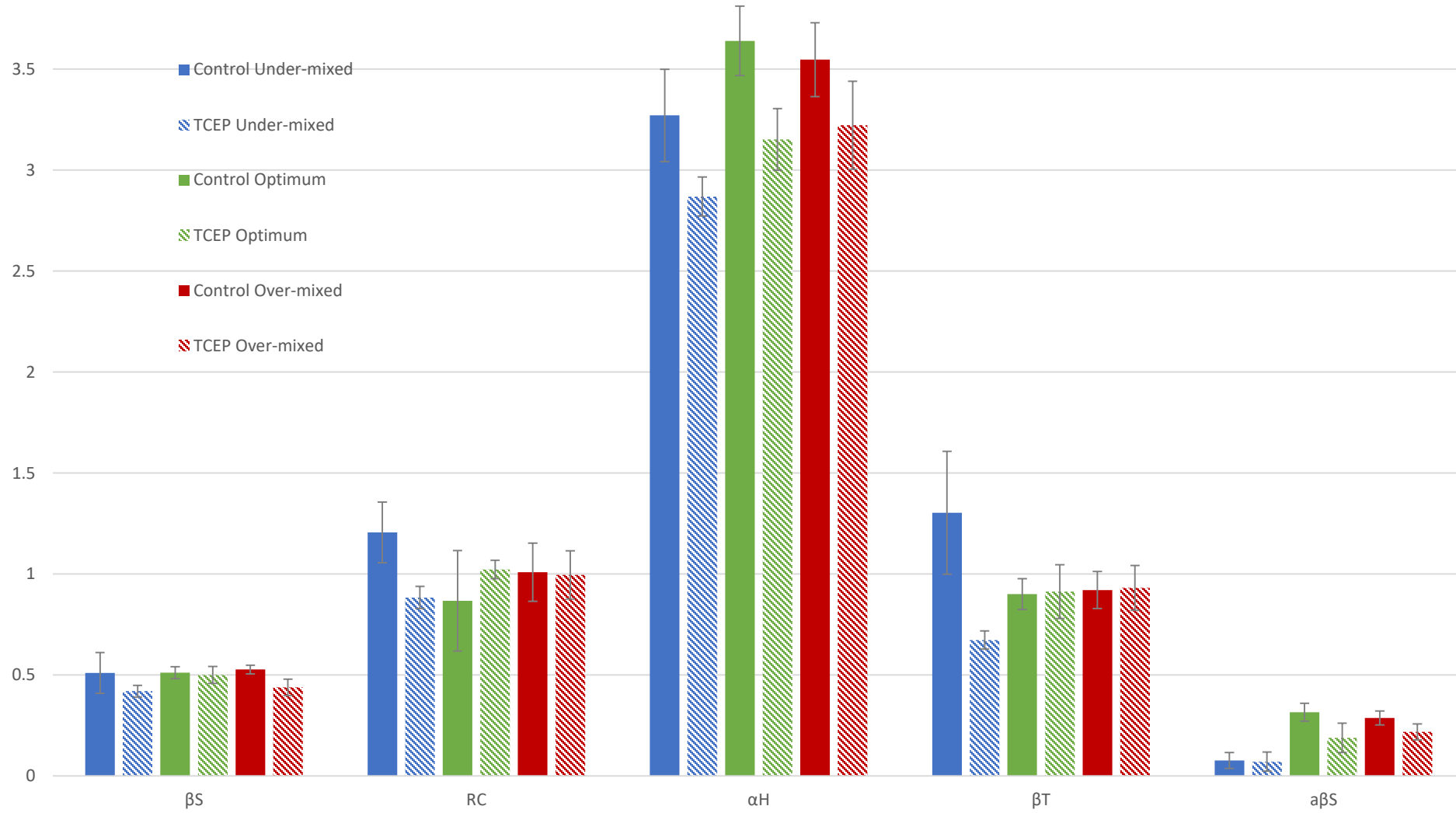
Figure 4: Raman determination of Disulphide bridge conformation for Control dough and Dough + TCEP for the three different mixing levels: Under-mixing, Optimum and Over-mixing (g-g-g (inter SS) conformation, t-g-g (intra SS) conformation, t-g-g conformation and t-g-t conformation).

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.

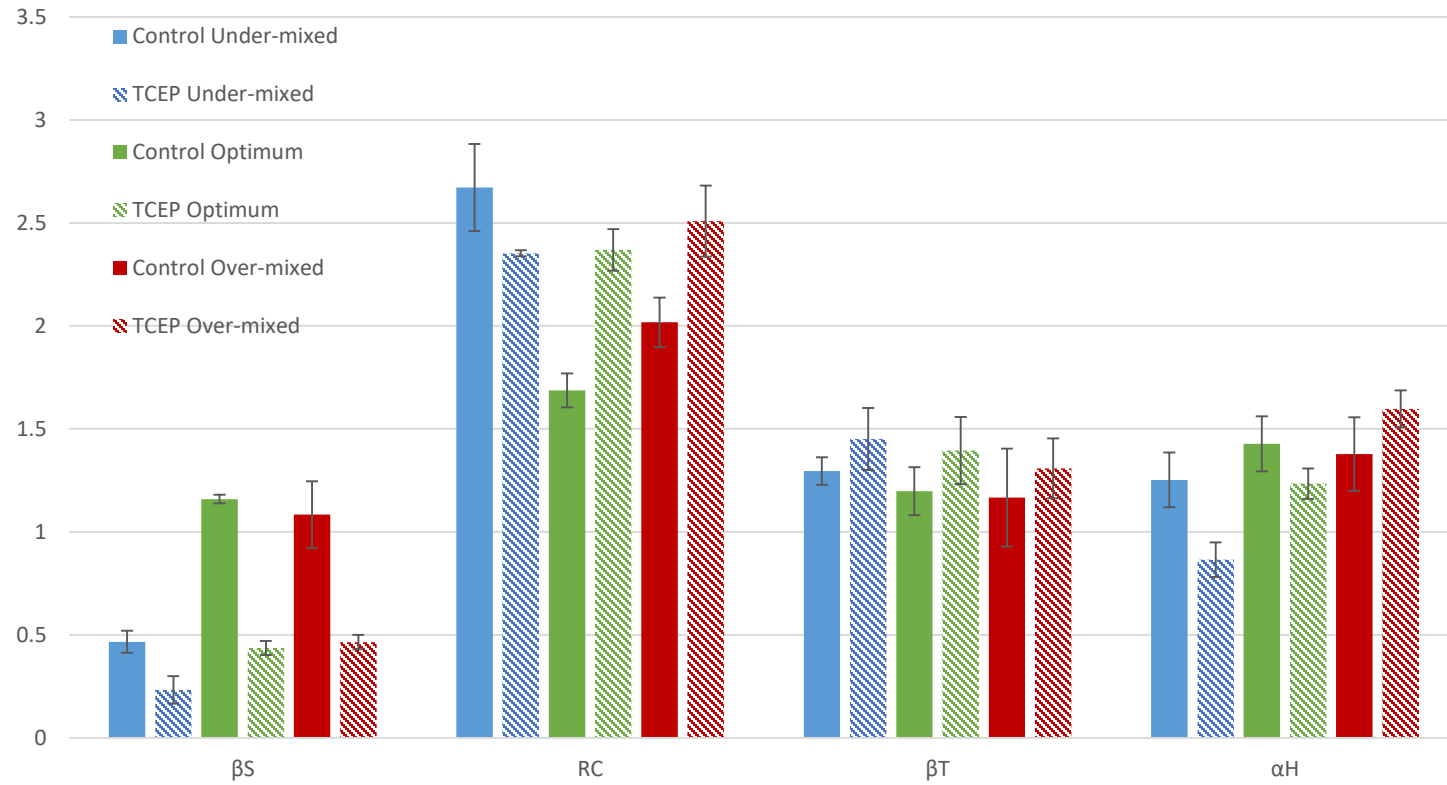
Figure 6: Raman determination of the Tryptophan doublet ratio for Control dough and Dough + TCEP for the three different mixing levels: Under-mixing, Optimum and Over-mixing.



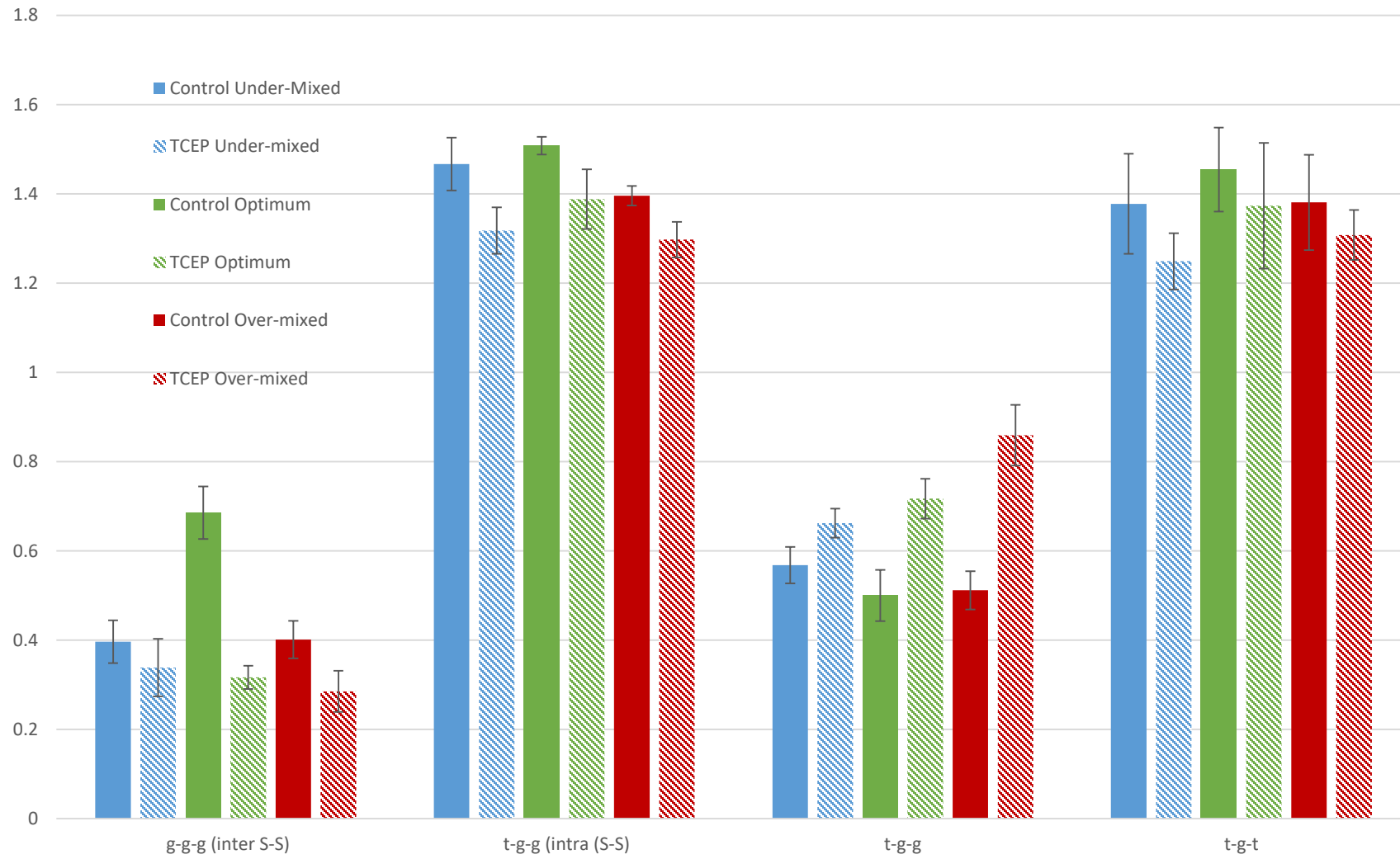
Amide I



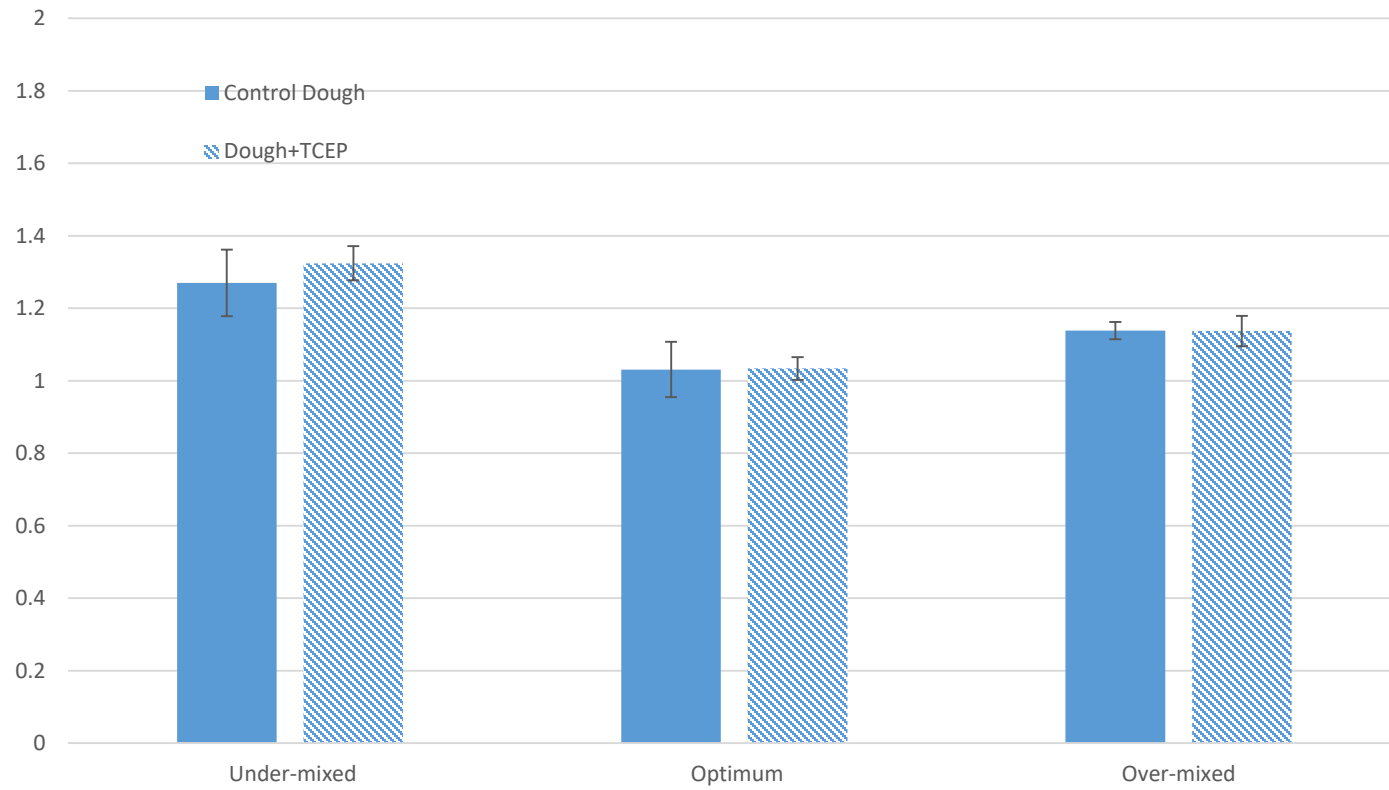
Amide III



Disulphide bonds



Tyrosine



Tryptophane

