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1 **Effects of ozone treatment on the molecular properties of wheat**
2 **grain proteins**

3
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29 **ABSTRACT**

30 Ozone is a powerful and highly reactive oxidizing agent, which has found increasing
31 applications in the field of grain processing. However, in some cases, O₃ can potentially
32 promote oxidation and/or degradation of the chemical constituents of grains. Experiments were
33 carried out to evaluate the specific effects of gaseous ozone on the molecular properties of
34 wheat grain proteins and their consequences on the bread-making quality of the resulting flours.

35 Ozonation causes a significant reduction in the SDS solubility of the wheat prolamins,
36 which can reasonably be attributed to conjugate effects of an increase in molecular dimensions
37 and an increase in the compactness of the protein polymers initially present. In fact, our results
38 demonstrate that this general reinforcement of the aggregative status of prolamins due to
39 ozonation of wheat grains results from (i) the formation of new intermolecular S-S bonds, (ii)
40 to a lesser extent, the formation of other types of intermolecular covalent cross-links (dityrosine
41 cross-links) and finally, (iii) significant changes in secondary structure. By significantly
42 affecting the molecular properties of wheat grain prolamins, ozone leads to profound changes
43 in the rheological properties (i.e. increase in the tenacity and a great limitation of the
44 extensibility) of the flours and/or doughs obtained.

45

46

47 **Keywords:**

48 Ozone treatment, Wheat grain proteins, Prolamin aggregative properties, Bread-making quality.

49 1. Introduction

50 Ozone, which was recognized in 1997 by the U.S. Food and Drug Administration (FDA)
51 as a GRAS (i.e. Generally Recognized as Safe) substance for use as a disinfectant or sanitizer
52 in the food industry (Alexandre et al., 2011), is a powerful and highly reactive oxidizing agent,
53 which has found increasing applications in many different fields. A recent review lists the
54 various applications of ozone in the field of grain processing (Tiwari et al., 2010). As reported
55 in this paper, which refers to the Oxygreen® process in particular, the ozonation of grains
56 responds to a general concern about food safety [i.e. a reduction in the microorganism and virus
57 load (Khadre et al., 2001), the control of various fungi (Wu et al., 2006; Savi et al., 2014) and
58 the elimination of different contaminants, such as residues of pesticides and mycotoxins in
59 particular (Trombete et al., 2016)].

60 Over the last few years, several studies have made it possible to define the general
61 conditions for using gaseous ozone in the field of cereal grain processing (Yvin et al., 2001;
62 Costes et al., 2005). For example, a pre-humidification stage to increase the moisture of the
63 grains to approximately 15 - 17 % has been proposed; the grains are ozonated with ozone
64 quantities between 5 and 10 g O₃.kg⁻¹ of grain; the ozone concentration in the carrier gas ranges
65 between 80 and 140 g O₃.m⁻³ TPN; the ozone pressure in the reactor used has been fixed at
66 between 250 and 650 mbars; and the duration of the treatment is about 30 - 180 min.

67 However, due to its strong oxidizing properties, ozone is not universally beneficial
68 because, in some cases, it can potentially promote degradation and/or depolymerization of the
69 main chemical constituents of grains (i.e. lipids, storage proteins, polysaccharides). Although
70 some authors (Sandhu et al., 2011; Violleau et al., 2012; Chittrakorn et al., 2014) have pointed
71 out that these chemical alterations can lead to modifications of the technological properties

72 (particularly rheological properties) of the treated materials, the precise molecular effects as
73 well as the associated mechanisms *in situ* are still very poorly documented.

74 In order to describe as well as control these mechanisms better, we have been studying
75 for some years the *in situ* effects of ozone gas on the native polymeric structures of wheat grain
76 (i.e. starch polysaccharides and storage proteins); polymeric structures that are largely
77 responsible for defining the technological capabilities of the products obtained after processing
78 (i.e. wheat flours and/or wheat doughs). Thus, in a recent study (Gozé et al., 2016), we
79 demonstrated that, whatever the soft wheat cultivar investigated, there is no significant effect
80 of gaseous ozone on wheat starch (in particular, no change in molecular weight distribution)
81 when the ozonation treatment is applied to whole grains (i.e. without any previous
82 transformation of the grains). Only slight increases in carboxyl groups were observed with
83 increasing ozone consumption.

84 These original results, which contradict some published works (Sandhu et al., 2012;
85 Klein et al., 2014), suggest that the main origin of the observed changes in the technological
86 properties of flours and/or doughs derived from ozonated grains could be the protein fraction
87 contained in the starchy endosperm (i.e. storage proteins mainly composed by gliadins and
88 glutenins).

89 In the present study, we investigated the specific effects of gaseous ozone on the
90 molecular properties of wheat grain proteins. This work made it possible to evaluate the impact
91 of the oxidizing treatment on (i) the solubility and the molecular weight distribution (MWD) of
92 these proteins, (ii) the thiol-disulfide status of polymeric proteins and the formation of new
93 inter-molecular cross-links, (iii) the protein secondary structure, and (iv) the rheological
94 properties of wheat flours.

95

96

97 2. Material and methods

98

99 2.1. Plant material

100 The two bread wheat cultivars used in this study were Rubisko and Diamento, differing
101 in their HMW-GS composition (7+8/2+12 and 7+9/5+10, respectively) (Certified seeds, harvest
102 2013, RAGT France).

103

104 2.2. Ozone treatment

105 All experiments were conducted with a specific reactor set up at the Institut
106 Polytechnique UniLaSalle. A 10 kg wheat grain mass was fed into a semi-batch stainless steel
107 reactor equipped with a central endless screw for grain circulation. The temperature in the
108 reactor was maintained at 22 °C by a cooling system. The reactor pressure was maintained at
109 0.3 bars by a gas vector. Ozone gas was fed into the bottom of the reactor through a micro-
110 porous plate with a flow rate of 2.0 m³ NTP.h⁻¹. Ozone was produced from either dry air (dew
111 point -70 °C) or oxygen (99.5 %, Air-liquid, France), which were connected to an ozone
112 generator (OZAT[®] CFS-2G, Ozonia, France). Inlet and outlet ozone concentrations were
113 continuously measured and recorded (Ozone Analyzer BMT 964). The ozone off gas was
114 destroyed at 350 °C by a thermal destruction unit.

115

116 2.3. Experimental design

117 In order to generate a wide range of wheat grain samples differentiated in terms of ozone
118 oxidation (i.e. amount of O₃ consumed), fifteen experiments with several combinations of (i)
119 pre-humidification level (coded as X_1) during preparation of the grain, (ii) ozone concentration

120 in the ozone inlet (coded as X_2) and (iii) reaction time (coded as X_3) were used as the main
121 factors and their levels are shown in Table 1A. A 3-factor 3-level factorial Box-Behnken (BB)
122 experimental design technique was employed (Table 1B) to investigate the effects of the
123 selected variables. BB designs are response surface methods (RSM), specially constructed to
124 require only 3 levels, coded as -1, 0, and +1. The results were analyzed using Statistica version
125 12.1 (Statsoft, France) with $P < 0.05$. Response surface analysis was used to estimate the model
126 coefficient and carry out a response surface regression (RSREG) procedure using the software.

127

128 2.4. *Sample preparation and total protein content determination*

129 After ozone treatment, 1 kg of each wheat sample was milled in a CD1 mill (CHOPIN
130 Technologies, France) with water adjustment (15.5 %, BIPEA). The total protein content (TPC)
131 of each sample was measured on 120 mg of oven-dried wheat flour by elementary nitrogen
132 analysis (DUMAS method - AOAC 7024) on a Leco apparatus (model FP528). Three replicates
133 were carried out and combined for analysis, using a conversion factor of $N \times 5.7$.

134

135 2.5. *Alveographic measurements*

136 Alveographic measurements were made following the NF ISO 5530-4 standard.

137

138 2.6. *Quantification and measurement of unextractable polymeric protein (UPP)*

139 Flour samples (120 mg) were dispersed and incubated at 60 °C for 2 hours with 12 mL
140 of 0.05 M sodium phosphate buffer (pH 6.9) containing 2 % (w/v) SDS, with constant stirring.
141 After centrifugation for 30 min at 12,500 $\times g$ at 20 °C, the supernatant, containing the

142 extractable fraction (EP), was removed. The pellets, containing the unextractable polymeric
143 protein (UPP), were frozen at -20 °C for 24 h, freeze-dried for 48 h and then their protein
144 contents were determined by the DUMAS method as previously described. The extractable
145 protein (EP) fraction was calculated from the formula $EP = TPC - UPP$.

146

147 2.7. *Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis*

148 Flour samples (50 mg) were dispersed and incubated at 60 °C for 2 h with 1 mL of 0.05
149 M sodium phosphate buffer (pH 6.9) containing 2 % (w/v) SDS and 1 % (w/v) dithiothreitol
150 (DTT). For non-reduced proteins, the extraction buffer did not contain DTT. The extracts were
151 then sonicated for 15 sec at a power setting of 30 % using a stepped microtip probe (3 mm
152 diameter) (Sonics Materials, Bioblock Scientific, model 75038) and centrifuged for 15 min at
153 $12,500 \times g$ at 25 °C. The protein extracts were then subjected to electrophoresis in vertical SDS-
154 PAGE slabs (Mini PROTEAN II cell, Bio-Rad Laboratories) at a gel concentration of 10 % in
155 a discontinuous, pH 6.8-8.8, Tris-Glycine-SDS buffer system. Sample volumes of 15 µl were
156 loaded into each well and electrophoresis was performed at 170 V during the run. After the
157 migration, the gel was fixed with 40 % ethanol and 10 % acetic acid (v/v) for 15 min with gentle
158 stirring and then stained for 15 h with 50 ml of QC Colloidal Coomassie stain. Deionized water
159 was used for gel destaining.

160

161 2.8. *FT-IR analysis*

162 A standard procedure was used for processing dried samples into KBr disks. Approximately
163 1 mg of dried matter was mixed with 300 mg of dry KBr powder using a pestle and mortar. The
164 resulting mixture was transferred into a stainless steel holder (13 mm inner diameter), which

165 was placed in a hydraulic press (RIIC-Beckmann, Glenrothes, Scotland) and evacuated with an
166 attached vacuum pump. The press was adjusted to 40000 kg, generating a pressure of
167 approximately 3000 kg.cm⁻² on the pellet within the holder. Pressure was maintained for 1 min.
168 Fourier-transform infrared (FT-IR) spectra were recorded with a Nicolet iZ10 FT-IR
169 Spectrometer (Nicolet, Madison, WI, USA), equipped with a sample wheel for the KBr pellet
170 deuterated triglycine sulfate (DTGS) detector. Three replicate spectra were collected at a
171 resolution of 2 cm⁻¹ co-adding 256 scans with a spectral range of 650 to 4000 cm⁻¹.

172 Absorbance spectra were converted into printable format by the OMNIC8 software
173 package, and then imported into MATLAB for further analysis. Calculations were performed
174 using scripts written by the authors and free downloadable MATLAB functions for scientists
175 (<http://terpconnect.umd.edu/~toh/>). Because the protein amide I (bands at about 1656 cm⁻¹,
176 which is representative of protein secondary structures) component bands overlapped, a specific
177 multipeak fitting or modeling procedure was required. To determine the relative amounts of α -
178 helices, β -sheets, random coils and turns in the protein secondary structure, two steps were
179 applied. The first step was to use Fourier self-deconvolution (FSD) to reduce the width and
180 restore the intrinsic line-shape of the amide I band. The detailed concepts and algorithm of FSD
181 are described in Griffiths and Pariente (1986). The second step was to use a curve-fitting
182 program with a Gaussian function. A linear baseline was always used between 1600 and 1720
183 cm⁻¹ and the fourth derivative function was calculated to determine the number and positions
184 of the bands corresponding to the different components in the amide I profile. This function
185 was used instead of the second derivative function to increase the sensitivity of the peak
186 detection (Fleissner et al., 1996). In the curve-fitting process, the positions of the band
187 components were fixed, whereas their bandwidths could be adjusted to perform the curve-fitting
188 of the amide I profile. The assignment of individual components to secondary structural
189 elements according to this band decomposition was as follows : α -helix, 1655 cm⁻¹, 1659 cm⁻¹,

190 1664 cm^{-1} ; β -sheet, 1625 cm^{-1} , 1630 cm^{-1} , 1637 cm^{-1} , 1641 cm^{-1} , 1644 cm^{-1} , 1691 cm^{-1} ; β -turn,
191 1671 cm^{-1} , 1676 cm^{-1} , 1686 cm^{-1} ; and random coil 1648 cm^{-1} . The two components at 1612 cm^{-1}
192 and 1617 cm^{-1} were ascribed to protein side chains as these frequencies are too low to be
193 assigned to any secondary structure. The amount of each secondary structure element is given
194 in percentage terms, by dividing the area of one amide I band component by the area of the sum
195 of all amide I band component areas.

196

197 2.9. *Asymmetrical flow field flow fractionation (A-FFFF) system and procedure*

198 Samples were prepared according to the protocol of protein extraction, described in
199 detail by Lemelin et al. (2005). Briefly, flour samples (30 mg) were dispersed and incubated at
200 60 °C for 15 min with 1.0 mL of 0.05 M sodium phosphate buffer (pH 6.9) containing 2 %
201 (w/v) SDS. The extracts were then sonicated for 20 sec at a power setting of 30 %. The
202 supernatant (centrifugation at 12,500 \times g at 20 °C for 15 min) was filtered through 0.45 μm
203 filters (Gelman Sciences, France) before injection (30 μl) into the AFFFF/MALLS system.
204 Protein content was determined for each extract by combustion nitrogen analysis as described
205 above. AF4 was accomplished with an Eclipse3 F System (Wyatt Technology, Santa Barbara,
206 CA, USA) serially connected to a UV detector (Agilent 1200, Agilent Technologies, Germany),
207 a MALLS detector (Dawn multi-angle Heleos TM, Wyatt Technology Corporation, Europe)
208 and an interferometric refractometer (Optilab rEX, Wyatt Technology Corporation, Europe).
209 Absorbance was measured at 214 nm. The channel had a trapezoidal geometry and a length of
210 286 mm. The thickness of the spacer used in this experiment was 350 mm. The ultrafiltration
211 membrane forming the accumulation wall was made of regenerated cellulose with a cut-off of
212 10 kDa (LC-10 Nadir reg. Cell, Wyatt Technology Europe, Germany). An Agilent 1200 Series
213 Isocratic HPLC Pump (Agilent Technologies, Germany) with an in-line vacuum degasser
214 delivered the carrier flow to the channel. A 0.45 μm in-line filter (Gelman Sciences, France)

215 was installed between the main pump and the Eclipse system. Sodium phosphate buffer (0.05
216 M, pH 6.9) containing 0.1 % (w/v) SDS was used as the mobile phase and filtered through a
217 0.1 mm membrane (Gelman Sciences, France). For the fractionations using a gradient in the
218 cross-flow, the focus time was 0.5 min at a flow rate of 2 ml.min⁻¹, followed by a focus/injection
219 time of 1.0 min at 0.2 ml.min⁻¹ and a relaxation/focusing time of 0.5 min. Elution then followed
220 at an outflow rate (F_{out}) of 1.0 ml.min⁻¹ and with a cross-flow rate (F_c) decreasing exponentially
221 from 3.0 to 0.0 ml.min⁻¹ for 14 min. Finally, elution at a cross-flow rate of 0.0 ml/min was
222 maintained for 9 min.

223 All necessary constants for molecular weight distribution calculation were determined
224 previously (Lemelin et al., 2005). A bovine serum albumin (BSA) standard was used to
225 normalize the diodes of the MALLS detector. The calculated MW of BSA was reasonably close
226 to the nominal value.

227

228 2.10. Determination of glutenin free sulfhydryl (SH) groups

229 The procedure used was performed according to Rakita et al. (2014) with some
230 modifications. Wheat flour samples (300 mg) were stirred for 30 min at room temperature (25
231 °C) with 4 mL of 0.05 M phosphate buffer (pH 6.9) containing 50 % (v/v) propanol-1.
232 Extraction was followed by centrifugation (5,000 × g for 3 min). The supernatant, mainly
233 containing monomeric proteins, was removed. The pellet was then suspended in 1 ml of a
234 GluHCl/Tris-Gly solution (pH 8.0), vortexed for 5 min and then centrifuged at 12,500 × g for
235 15 min. 600 µl of a GluHCl/Tris-Gly solution was added to 400 µl of the supernatant and then
236 mixed with 250 µl of Ellman's reagent [solution of 40 mg of DTNB (5,50-dithiobis-2-
237 nitrobenzoic acid) in 10 ml of Tris-Gly buffer pH 8.0, freshly prepared before use]. The

238 absorbance was read at 412 nm. Results were calculated against an L-glutathione (G6529;
239 Sigma Aldrich) reduced standard curve (within the range 0.018 - 0.30 $\mu\text{mol.mL}^{-1}$).

240

241 2.11. *Quantification of dityrosine cross-links in the glutenin fraction*

242 The procedure used was performed according to Rodriguez-Mateos et al. (2006) with
243 some modifications. Wheat flour samples (200 mg) were placed in tubes with 5 mL of 6 N HCl
244 containing 1 % (w/v) of phenol and incubated at 110 °C for 24 h. Next, tubes were placed in a
245 Genevac EZ-2 and EZ-2plus evaporation system until total evaporation of the HCl and phenol.
246 Samples were then reconstituted in 0.4 mL of HPLC water containing 0.1 % (v/v) trifluoroacetic
247 acid (TFA), filtered through a 0.45 μm nylon filter, and loaded (25 μL) into the HPLC system.

248 A Thermo Separation Products HPLC system (Thermo Electron Corporation,
249 Courtaboeuf, France) was used in conjunction with a C-18 column Ace 5 AQ (25 cm \times 4.6 mm
250 i.d., Hichrom, Theale, Berkshire, United Kingdom) preceded by a guard column of the same
251 packing material. A stepwise gradient with acetonitrile and water containing 0.1 % (v/v) TFA
252 was used [3, 10, 40, 95, and 95 and 3 % (v/v) acetonitrile at 0, 35, 50, 60, 65, and 85 min,
253 respectively]. Column temperature was maintained at 30 °C and the flow rate was 0.7 mL/min.
254 A fluorescence detector at $\lambda_{\text{ex}} = 285$ nm and $\lambda_{\text{em}} = 405$ nm was used. Results were calculated
255 against a purified standard (D494290; Toronto Research Canada) curve (within the range 0.00
256 - 0.40 $\mu\text{g.mL}^{-1}$).

257

258 3. Results

259

260 3.1. *SDS protein solubility and molecular weight distribution (MWD) of polymeric proteins*

261 As can be seen in Table 2, the experimental design and the variables selected during this
262 experiment (particularly pre-humidification level and ozone concentration in the ozone inlet)
263 generated a wide range of oxidation of the samples; the latter was estimated on the basis of the
264 total quantity of gaseous ozone consumed by the wheat grains during their processing. Thus,
265 whatever the chemical composition of the plant material used (Diamento or Rubisko), the
266 treatment rate of the grains obtained varied from 0.00 to 16.11 g O₃.kg⁻¹ and 0.00 to 18.09 g
267 O₃.kg⁻¹ for Diamento and Rubisko, respectively.

268 The ozone treatment significantly modified the 2 % SDS solubility of flour proteins,
269 which was evaluated by the UPP/EP ratio. For example, for the cultivar Rubisko, this ratio
270 increased from 0.34 to 0.58 for the control and run17 (maximum O₃ consumption), respectively.
271 The same observation was made with the cultivar Diamento (increase in UPP/EP ratio from
272 0.63 to 0.97 for the control and run17, respectively). Initial differences in the UPP/EP ratio
273 between the two wheat genotypes (0.34 vs. 0.63 for Rubisko and Diamento, respectively) were
274 due to the allelic variations coded in particular by the *Glu-1D* locus (Carceller and Aussenac,
275 2001). In fact, the presence of the 5 and 10 HMW-GS from Diamento decreased the initial 2 %
276 SDS solubility of glutenins in contrast to the 2 and 12 HMW-GS from Rubisko.

277 At the same time, the ozone treatment significantly modified the MWD of flour proteins,
278 which was mainly evaluated by the number-average molar mass (M_n) and the weight-average
279 molar mass (M_w). Both M_n and M_w of the polymeric proteins significantly increased [from
280 9.38×10^5 g.mol⁻¹ and 3.81×10^7 g.mol⁻¹ to 15.34×10^5 g.mol⁻¹ and 6.77×10^7 g.mol⁻¹ for M_n and
281 M_w of the control and run17 (maximum O₃ consumption), respectively] as a result of the grain
282 ozonation. The same results were observed with the other hydrodynamic parameters, such as
283 the number-average radii of gyration (R_n) and the weight-average radii of gyration (R_w).

284 In Table 2, ν represents the dependence of size upon mass for the total glutenin polymers
285 of Diamento and Rubisko (i.e. the value obtained by fitting the linear relationship of the
286 logarithmic form of $R = k.M^\nu$). This linear relationship can be considered an estimation of the
287 molecular conformation of the studied polymers. As can be seen, ozonation led to a decrease in
288 this hydrodynamic parameter ν (from 0.34 to 0.29 and from 0.30 to 0.25 for the control and
289 run17 of Diamento and Rubisko glutenin polymers, respectively). This decrease indicates that
290 the molecular dimensions increase only slowly with the molecular weight and thus the
291 molecules have a more compact structure.

292 Finally, very strong quantitative relationships were observed between all the
293 macromolecular features of polymeric proteins (i.e. UPP/EP, M_w , M_n , R_n , R_w and ν) and the
294 amount of O_3 consumed by the wheat grains during the different ozone treatments (Table 2A
295 and 2B).

296

297 3.2. *SDS-PAGE profiles of wheat protein and formation of new protein cross-links*

298 Both non-reduced (Figure 1A) and reduced (Figure 1B) SDS-PAGE were used to
299 analyze the protein pattern changes in wheat flour after exposure to ozone gas. Proteins under
300 non-reducing conditions did not change remarkably due to the ozone treatment (Figure 1A).
301 Polymerization (bands with a $M_w \geq 250$ kDa on the top of the gel) seemed to occur mainly
302 through disulfide bonds, as evidenced by the reducing protein patterns shown in Figure 1B, in
303 which there was no major difference in most of the visible bands among all samples.

304 On the other hand, the data in Table 2 showed that the free SH-group content in
305 polymeric proteins decreased significantly (loss of ≈ 30 % and ≈ 40 % of the initial free -SH
306 content for Diamento and Rubisko, respectively) as ozone treatment intensity increased (i.e.
307 amount of O_3 consumed) indicating a potential formation of disulfide bonds in these proteins.

308 On the top of the reduced separated gel (Figure 1B), an increase in the intensities of bands from
309 the ozone-treated samples was also observed. Thus, high molecular mass aggregates were
310 formed even after the reduction of DTT. These results, which are in total agreement with
311 previous observations (Linlaud et al., 2011; Li et al., 2012), suggest that the cross-linking did
312 not only occur through disulfide bonds.

313 Table 3 shows the dityrosine concentrations present in the wheat grains after the
314 ozonation treatment. The initial levels obtained (i.e. control samples) in this study are in
315 excellent agreement with those observed in previous studies (Hanft and Koehler, 2005;
316 Rodriguez-Mateos et al., 2006) when expressed as a proportion of the mass of material assessed.
317 The treatment of the same wheat grains with gaseous ozone led to a significant increase in
318 dityrosine concentration in wheat grain storage proteins (from 0.499 to 0.821 nmol.g⁻¹ of flour
319 and from 0.561 to 0.733 nmol.g⁻¹ of flour for the control and run17 of Diamentio and Rubisko,
320 respectively). These results, which emphasize the formation of new protein cross-links in
321 response to the action of gaseous ozone, are perfectly consistent with our previous observations
322 (see the comments above on Figure 1B) and the suggestions of some authors (Verweij et al.,
323 1982).

324

325 3.3. *Protein secondary structure modifications*

326 To evaluate the effect of gaseous ozone on the secondary structure of proteins present
327 in the wheat grains, the FT-IR spectra of these proteins (i.e. mainly gliadins and glutenins) were
328 carried out. Quantitative analysis of protein secondary structure is based on the assumption that
329 protein can be considered a linear sum of a few fundamental secondary structural elements. The
330 α -helix, β -sheet, β -turn, and aperiodic structures, when taken together, constitute the secondary
331 structure of the protein backbone.

332 The relative contents of the β -sheet, random coil structure, α -helix and β -turn obtained
333 from the second derivative spectrum are summarized in Table 4. The FT-IR data reveal that the
334 amounts of α -helix and β -turn decreased relative to β -sheet as a function of ozone treatment.
335 As can be seen, ozonation led to a decrease in the ratio of α -helix to β -sheet (from 1.05 to 0.80
336 and from 1.08 to 0.70 for the control and run17 for Diamento and Rubisko glutenin polymers,
337 respectively). For the two wheat cultivars studied, this important ratio (α -helix/ β -sheet)
338 decreased as the molecular mass of the glutenin polymers/aggregates increased (Table 2). These
339 results, which are in agreement with previous works (Popineau et al., 1994), confirm that
340 intermolecular β -sheet structures may be involved in protein-protein interactions (i.e. the
341 formation of β -sheet structures by interactions between the glutenin repetitive domains) in
342 addition to intermolecular thiol/disulfide (SH/S-S) interchange or thiol/thiol (SH/SH) oxidation
343 reactions. Finally, the decrease in β -turn structures could be attributed to the possible oxidation
344 of the amino acid residues or to their cross-linking (for example, the formation of dityrosine
345 cross-links, whose presence was demonstrated above).

346

347 3.4. *Rheological properties of wheat flours*

348 Table 5 presents a summary of the major viscoelasticity properties of the flours (i.e.
349 tenacity, elasticity and extensibility) obtained from ozonated wheat grains and evaluated by
350 alveographic measurements. Regardless of the wheat cultivar studied, all flours made from
351 ozonated wheat grains had W values lower than the initial W value (from 169 to 63×10^{-4} J and
352 from 114 to 58×10^{-4} J for the control and run17 of Diamento and Rubisko, respectively). These
353 values were significantly and negatively correlated ($r = -0.866$ and -0.718 for Diamento and
354 Rubisko, respectively) with the amount of O_3 consumed by the wheat grains during their
355 treatment.

356 At the same time, the P/L values of all flours from ozonated grains showed higher values
357 than the control (from 1.89 to 5.67 and from 0.83 to 4.22 for Diamento and Rubisko,
358 respectively) and these values were significantly and positively correlated ($R = +0.605$ and
359 $+0.859$ for Diamento and Rubisko, respectively) with the amount of O_3 consumed.

360 These results clearly show that the treatment of wheat grains with gaseous ozone leads
361 to profound changes in the rheological properties of the different flours. These modifications,
362 apparently very strongly stoichiometric, result in a very large increase in the tenacity of the
363 doughs and a very great limitation of their extensibility.

364

365 **4. Discussion**

366 Wheat storage proteins are recognized as the most important components governing
367 bread-making quality (Schofield and Booth, 1983). To understand more precisely the
368 fundamental basis of the proteins that determine bread-making properties, such as mixing time,
369 extensibility and loaf volume, much attention has been focused on the (polymeric) glutenin
370 fraction. Both the molecular structure and functional properties of glutenin and its subunits have
371 been extensively reviewed (Weegels et al., 1996). Although the precise structure of glutenin is
372 still unknown, it has been shown that these proteins are multiple chain polymers in which the
373 individual polypeptides or subunits are mainly linked by disulfide bonds (Shewry and Tatham,
374 1990) and lead to large molecules with molecular weights ranging from a few hundred thousand
375 to many millions of Daltons (Carceller and Aussenac, 2001). The size of these polymeric
376 proteins is mainly determined by the genetically controlled composition of the high and low
377 molecular weight glutenin subunits (HMW-GS and LMW-GS) (Southan and MacRitchie,
378 1999) and the amount of the most strongly aggregating/largest glutenin polymers in flour is a
379 good measure of bread-making potential. In fact, a strong relationship has been established
380 between dough strength and/or baking quality and the average molecular size or size

381 distribution (MWD) of the polymeric protein components (Gupta et al., 1993). Consequently,
382 any alteration of the molecular dimensions and/or the aggregation status of these polymeric
383 proteins could potentially lead to a modification of the technological properties of the products
384 concerned. In our program, we studied the specific action of gaseous ozone on the molecular
385 properties (i.e. molecular dimensions and aggregative properties) of the reserve proteins of soft
386 wheat grains.

387 First, ozonation causes a significant reduction in the SDS solubility of the proteins
388 contained in the albumen of soft wheat grains. This decrease in solubility results in a significant
389 modification of the characteristic UPP/EP ratio, regardless of the initial protein composition
390 (i.e. nature and relative amount of the various protein subunits). All these modifications are
391 strongly correlated with the quantities of ozone consumed by the wheat grains during their
392 treatment, thus highlighting a significant quantitative relationship between these variables.

393 At the same time, the ozonation of the grains causes major changes in the different
394 molecular dimensions that are characteristic of the storage proteins. Thus, ozone generates a
395 significant increase in the average molecular masses (M_w and M_n) and in the radius of gyration
396 (R_w and R_n) of prolamins in general and glutenin polymers in particular. Even if, as in the
397 previous case, these modifications of molecular dimensions are strongly dependent on the
398 quantities of ozone consumed by the grains, contrary to the conclusions of Violleau et al.
399 (2012), the fact remains that the response curves obtained highlight a non-negligible genotypic
400 effect. This effect is due to the significant structural differences between the glutenin polymers
401 originally present in the selected varieties. These structural differences are related to the fact
402 that the protein assemblages were initially constructed from “base bricks” (i.e. HMW and LMW
403 glutenin subunits) with very different reactivity (i.e. different ability to form protein inter-
404 and/or intramolecular interactions). In fact, the quantitative effects of ozone on the molecular
405 dimensions of glutenin polymers can be strongly modulated by the initial structure of the latter.

406 The observed changes in the molecular distribution of glutenin polymers are
407 accompanied by discrete but very real variations in the conformation of these protein
408 assemblages. Thus, the various applied ozonation treatments induce a general increase in the
409 compactness of the structures, which is independent of the initial structure of the polymers
410 present before the oxidative treatment. This increase in compactness probably results from an
411 increase in the density of the initial protein network, resulting from the formation of novel
412 protein-protein interactions under the effect of the oxidizing agent.

413 It seems that we can reasonably attribute the changes in solubility observed in the grain
414 prolamins resulting from the ozonation treatment to the conjugate effects of an increase in
415 molecular mass and an increase in compactness of the protein assemblies present. In order to
416 understand better the role of ozone in these modifications, we naturally studied the impact of
417 this oxidizing agent on the aggregative status of glutenin polymers.

418 The vast majority of the glutenin polymers formed in wheat grain result from the
419 intermolecular association of the various constituent subunits (i.e. LMW and HMW glutenin
420 subunits) via S-S covalent bridges formed from some free -SH groups carried on the cysteine
421 residues present almost exclusively in the -C and -N terminals of these polypeptides. At the
422 same time, ozonation of wheat grains causes (i) the formation of new intermolecular S-S bonds
423 by strongly mobilizing -SH groups still free at the protein subunits, (ii) to a lesser extent, the
424 formation of other types of intermolecular covalent bridges involving highly represented amino
425 acid residues in the repeatable domains of the protein subunits (tyrosine residues, for example)
426 and (iii) significant changes in secondary structure (i.e. reduction in the ratio of α -helices to β -
427 sheet) reflected, in particular, by the reinforcement of intermolecular β -sheet type structures
428 from the same protein repeatable domains.

429 Taken together, these results demonstrate that the ozonation of wheat grains causes a
430 significant reinforcement of the aggregative status of prolamins in general and glutenin
431 polymers in particular. Thus, all the mechanisms (covalent or not) generated at the end of an
432 ozonation treatment contribute to strengthening the protein-protein interactions and will
433 consequently lead to changes in the molecular dimensions and compactness of the protein
434 assemblies; themselves responsible for the loss of solubility (i.e. 2 % SDS solubility) observed.
435 As we have demonstrated in this study, by significantly affecting the molecular properties (i.e.
436 molecular dimensions, conformation, aggregative properties) of wheat grain prolamins, ozone
437 leads to profound changes in the rheological properties (i.e. increase in the tenacity and a great
438 limitation of the extensibility) of the flours and/or doughs obtained after the ozonation
439 treatment.

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441 **5. Conclusions**

442 As a result of this experimental work, it is clear that although the ozonation process is
443 currently being used in the field of cereal processing mainly to meet food safety requirements,
444 ozone may led to a modification of the majority of the techno-functional properties of processed
445 products (i.e. flours and/or doughs). As our results show, these changes in techno-functional
446 properties are mainly due to changes in the aggregate status of prolamins in grain. Given the
447 action of ozone on the aggregative status of prolamins, the technological implementation of the
448 flours generated requires specific and essential adjustments so that the professional can take
449 full advantage of their new functionalities. Finally, the effect of gaseous O₃ on the other grain
450 constituents (i.e. arabinoxylans and β -glucans) is currently being investigated.

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455 this research.

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564 **Figure captions**

565

566 **Fig. 1.** SDS-PAGE analysis of total protein patterns in flour of two bread wheat cultivars [(R)
567 Rubisko and (D) Diamento] with or without (T) ozone treatment. (A) Non-reduced patterns and
568 (B) reduced patterns. Lanes 18 and 17 (replicates 1 and 2) correspond to different ozone
569 treatments [coded values (-1/-1/-1) and (+1/+1/+1), respectively]. (Sd) Molecular weight
570 standards.

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Table 1. (A) Relationship between coded and uncoded levels of variables (B) Coded values used in the 3-factor 3-level factorial Box-Behnken experimental design.

| Coded level | Uncoded level of variables | | |
|-------------|---|--------------------------------------|--------------------|
| | Humidification level | Inlet ozone concentration | Reaction time |
| | (X_1) [H ₂ O % (v/w)] | (X_2) (g.m ⁻³ NTP) | (X_3) (min) |
| +1 | 6.0 | 180.0 | 60.0 |
| 0 | 4.0 | 120.0 | 37.5 |
| -1 | 2.0 | 60.0 | 15.0 |

| Run | Humidification level (X_1) [H ₂ O % (v/w)] | Inlet ozone concentration (X_2) (g.m ⁻³ NTP) | Reaction time (X_3) (min) |
|-----------------|--|--|----------------------------------|
| 1 | 0 | -1 | -1 |
| 2 | +1 | 0 | -1 |
| 3 | 0 | +1 | -1 |
| 4 | -1 | 0 | -1 |
| 5 | -1 | -1 | 0 |
| 6 | +1 | -1 | 0 |
| 7 | +1 | +1 | 0 |
| 8 | -1 | +1 | 0 |
| 9 | 0 | -1 | +1 |
| 10 | +1 | 0 | +1 |
| 11 | 0 | +1 | +1 |
| 12 | -1 | 0 | +1 |
| 13 | 0 | 0 | 0 |
| 14 | 0 | 0 | 0 |
| 15 | 0 | 0 | 0 |
| 17 ^a | 1 | 1 | 1 |
| 18 ^a | -1 | -1 | -1 |

^(a) Extra runs.

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Table 2. Macromolecular features of polymeric proteins in ozone-treated grains of two bread wheat cultivars [(A) Diamento and (B) Rubisko].

| Run | Diamento | | | | | | | |
|----------------------|--|---------------------|--|--|-------------------------------------|-------------------------------------|----------------|--|
| | O ₃ cons. (g.kg ⁻¹ grain) | UPP/EP ^a | M _w ^a (×10 ⁷ g.mol ⁻¹) | M _n ^a (×10 ⁵ g.mol ⁻¹) | R _n ^a (nm) | R _w ^a (nm) | v ^b | [free SH] ^a (μmol.g ⁻¹ glutenin) |
| T | 0.00 | 0.64 | 2.55 | 9.63 | 53.07 | 79.50 | 0.34 | 6.72 |
| 1 | 2.16 | 0.76 | 3.45 | 9.96 | 56.05 | 82.92 | 0.32 | 6.36 |
| 2 | 4.01 | 0.67 | 3.52 | 10.56 | 58.27 | 85.22 | 0.31 | 5.61 |
| 3 | 4.97 | 0.84 | 3.67 | 11.04 | 62.40 | 88.06 | 0.31 | 5.89 |
| 4 | 2.57 | 0.67 | 3.52 | 10.12 | 56.90 | 84.37 | 0.32 | 5.85 |
| 5 | 2.79 | 0.63 | 3.55 | 10.34 | 55.63 | 85.83 | 0.31 | 5.46 |
| 6 | 4.84 | 0.68 | 4.66 | 11.11 | 63.26 | 91.36 | 0.29 | 5.29 |
| 7 | 11.12 | 0.98 | 6.46 | 11.50 | 61.56 | 96.30 | 0.30 | 4.61 |
| 8 | 6.73 | 0.73 | 3.82 | 10.90 | 59.32 | 86.35 | 0.30 | 5.46 |
| 9 | 6.28 | 0.82 | 4.42 | 10.95 | 60.62 | 90.50 | 0.30 | 5.47 |
| 10 | 12.28 | 0.78 | 5.41 | 12.45 | 65.27 | 98.95 | 0.29 | 5.07 |
| 11 | 14.51 | 0.74 | 4.94 | 12.16 | 64.47 | 97.00 | 0.30 | 5.00 |
| 12 | 6.49 | 0.67 | 3.62 | 10.59 | 57.20 | 84.83 | 0.31 | 5.22 |
| 13 | 7.87 | 0.78 | 4.61 | 11.06 | 61.03 | 91.50 | 0.30 | 4.67 |
| 14 | 7.97 | 0.67 | 4.32 | 11.20 | 62.27 | 90.10 | 0.30 | 5.64 |
| 15 | 7.54 | 0.74 | 4.53 | 11.03 | 63.26 | 92.83 | 0.30 | 5.01 |
| 17 | 16.11 | 0.89 | 5.98 | 12.92 | 66.50 | 101.6 | 0.29 | 5.07 |
| 18 | 1.42 | 0.65 | 3.64 | 10.05 | 55.90 | 84.90 | 0.31 | 6.30 |
| <i>r^c</i> | 1.00 | 0.625* | 0.863* | 0.961* | 0.860* | 0.934* | --- | -0.764* |
| Run | Rubisko | | | | | | | |
| | O ₃ cons. (g.kg ⁻¹ grain) | UPP/EP ^a | M _w ^a (×10 ⁷ g.mol ⁻¹) | M _n ^a (×10 ⁵ g.mol ⁻¹) | R _n ^a (nm) | R _w ^a (nm) | v ^b | [free SH] ^a (μmol.g ⁻¹ glutenin) |
| T | 0.00 | 0.34 | 3.81 | 9.38 | 55.30 | 80.00 | 0.30 | 9.21 |
| 1 | 1.91 | 0.41 | 3.61 | 9.27 | 56.17 | 78.17 | 0.29 | 6.73 |
| 2 | 3.91 | 0.44 | 3.79 | 8.65 | 61.95 | 66.40 | 0.28 | 6.38 |
| 3 | 5.40 | 0.49 | 4.30 | 9.52 | 62.65 | 71.07 | 0.28 | 6.98 |
| 4 | 2.86 | 0.43 | 4.82 | 11.56 | 68.63 | 87.93 | 0.27 | 7.50 |
| 5 | 3.12 | 0.44 | 3.73 | 10.52 | 50.95 | 77.35 | 0.29 | 6.71 |
| 6 | 5.18 | 0.55 | 3.70 | 10.38 | 58.86 | 80.40 | 0.29 | 5.87 |
| 7 | 11.50 | 0.63 | 5.92 | 12.68 | 68.62 | 95.90 | 0.27 | 5.80 |
| 8 | 6.38 | 0.48 | 4.44 | 12.56 | 71.25 | 88.25 | 0.27 | 5.95 |
| 9 | 6.65 | 0.51 | 4.30 | 12.14 | 62.56 | 78.56 | 0.27 | 6.14 |
| 10 | 12.47 | 0.71 | 4.55 | 14.71 | 79.00 | 96.66 | 0.28 | 5.30 |
| 11 | 13.62 | 0.62 | 4.73 | 11.40 | 71.20 | 83.20 | 0.27 | 5.38 |
| 12 | 6.06 | 0.48 | 3.59 | 12.44 | 66.23 | 82.76 | 0.26 | 6.06 |
| 13 | 7.84 | 0.52 | 3.62 | 10.15 | 58.30 | 80.15 | 0.27 | 6.20 |
| 14 | 7.76 | 0.57 | 4.07 | 12.26 | 72.33 | 88.43 | 0.26 | 6.18 |
| 15 | 7.13 | 0.56 | 4.42 | 11.83 | 65.33 | 87.93 | 0.24 | 6.35 |
| 17 | 18.09 | 0.58 | 6.77 | 15.34 | 72.36 | 98.40 | 0.25 | 5.89 |
| 18 | 1.65 | 0.41 | 4.45 | 10.37 | 62.60 | 90.40 | 0.24 | 7.88 |
| <i>r^c</i> | 1.00 | 0.850* | 0.720* | 0.776* | 0.698* | 0.578* | --- | -0.735* |

596 ^(a) Mean value of three experiments (n = 3); ^(b) Conformation plot slope; ^(c) Regression
 597 coefficient – (*) significant up to 1 % level.

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Table 3. Dityrosine concentration in flours from ozonated grain of two wheat genotypes (Diamento and Rubisko).

| Run | Diamento | | | | Rubisko | | | |
|-----|---|-----------------|---|-----------------|---|-----------------|---|-----------------|
| | Dityrosine ($\mu\text{g}\cdot\text{g}^{-1}\text{flour}$) | | Dityrosine ($\text{nmol}\cdot\text{g}^{-1}\text{flour}$) | | Dityrosine ($\mu\text{g}\cdot\text{g}^{-1}\text{flour}$) | | Dityrosine ($\text{nmol}\cdot\text{g}^{-1}\text{flour}$) | |
| | Mean ^a | SD ^b | Mean ^a | SD ^b | Mean ^a | SD ^b | Mean ^a | SD ^b |
| T | 0.180 | 0.012 | 0.499 | 0.032 | 0.202 | 0.002 | 0.561 | 0.005 |
| 1 | 0.268 | 0.010 | 0.744 | 0.027 | 0.189 | 0.017 | 0.624 | 0.046 |
| 2 | 0.246 | 0.011 | 0.682 | 0.030 | 0.182 | 0.017 | 0.505 | 0.046 |
| 3 | 0.245 | 0.030 | 0.680 | 0.081 | 0.178 | 0.009 | 0.493 | 0.024 |
| 4 | 0.268 | 0.013 | 0.743 | 0.035 | 0.228 | 0.021 | 0.632 | 0.057 |
| 5 | 0.261 | 0.019 | 0.722 | 0.051 | 0.168 | 0.007 | 0.465 | 0.019 |
| 6 | 0.191 | 0.014 | 0.528 | 0.038 | 0.213 | 0.022 | 0.590 | 0.059 |
| 7 | 0.246 | 0.010 | 0.686 | 0.027 | 0.213 | 0.006 | 0.591 | 0.016 |
| 8 | 0.254 | 0.033 | 0.704 | 0.090 | 0.244 | 0.004 | 0.676 | 0.011 |
| 9 | 0.392 | 0.028 | 1.087 | 0.076 | 0.207 | 0.022 | 0.574 | 0.059 |
| 10 | 0.239 | 0.017 | 0.663 | 0.046 | 0.228 | 0.010 | 0.631 | 0.027 |
| 11 | 0.254 | 0.038 | 0.705 | 0.103 | 0.265 | 0.012 | 0.734 | 0.032 |
| 12 | 0.222 | 0.011 | 0.616 | 0.030 | 0.207 | 0.006 | 0.573 | 0.016 |
| 13 | 0.215 | 0.009 | 0.596 | 0.024 | 0.197 | 0.032 | 0.547 | 0.086 |
| 14 | 0.240 | 0.025 | 0.664 | 0.068 | 0.222 | 0.006 | 0.616 | 0.016 |
| 15 | 0.208 | 0.009 | 0.577 | 0.024 | 0.202 | 0.031 | 0.558 | 0.084 |
| 17 | 0.296 | 0.017 | 0.821 | 0.046 | 0.265 | 0.022 | 0.733 | 0.059 |
| 18 | 0.236 | 0.020 | 0.653 | 0.054 | 0.179 | 0.002 | 0.496 | 0.005 |

^(a) Mean value of five experiments (n = 3).

^(b) Standard Deviation.

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Table 4. Protein secondary structure (%) determined by Fourier transform infrared (FTIR) spectroscopy in flours from ozonated grain of two wheat genotypes (Diamento and Rubisko).

| Run | Diamento | | | | | | Rubisko | | | | | |
|----------------------|---|--------------------------|------------------------------|--------------------------|-------------------------|-----------------|---|--------------------------|------------------------------|--------------------------|-------------------------|-----------------|
| | O ₃ cons. (g.kg ⁻¹ grain) | β-sheet ^a (%) | Random coil ^a (%) | α-helix ^a (%) | β-turn ^a (%) | α-helix/β-sheet | O ₃ cons. (g.kg ⁻¹ grain) | β-sheet ^a (%) | Random coil ^a (%) | α-helix ^a (%) | β-turn ^a (%) | α-helix/β-sheet |
| T | 0.00 | 38.47 | 7.54 | 40.32 | 10.46 | 1.05 | 0.00 | 33.77 | 9.72 | 36.36 | 15.36 | 1.08 |
| 1 | 1.91 | 40.14 | 10.59 | 39.84 | 10.32 | 0.99 | 1.91 | 36.77 | 12.63 | 35.82 | 14.53 | 0.97 |
| 2 | 3.91 | 41.72 | 12.13 | 39.72 | 9.55 | 0.95 | 3.91 | 38.21 | 14.79 | 34.76 | 14.87 | 0.91 |
| 3 | 5.40 | 41.43 | 12.02 | 38.44 | 9.49 | 0.93 | 5.40 | 40.05 | 14.97 | 34.25 | 14.04 | 0.86 |
| 4 | 2.86 | 39.73 | 10.77 | 39.69 | 9.75 | 1.00 | 2.86 | 37.32 | 13.69 | 35.60 | 14.57 | 0.95 |
| 5 | 3.12 | 39.38 | 11.12 | 39.30 | 9.20 | 1.00 | 3.12 | 38.52 | 14.13 | 34.63 | 15.14 | 0.90 |
| 6 | 5.18 | 40.96 | 12.07 | 38.10 | 9.28 | 0.93 | 5.18 | 39.02 | 14.92 | 33.77 | 13.84 | 0.87 |
| 7 | 11.50 | 43.30 | 14.17 | 36.35 | 8.58 | 0.84 | 11.50 | 41.62 | 17.27 | 30.88 | 11.81 | 0.74 |
| 8 | 6.38 | 41.82 | 12.87 | 37.90 | 8.73 | 0.91 | 6.38 | 40.10 | 15.52 | 34.25 | 13.90 | 0.85 |
| 9 | 6.65 | 41.61 | 13.10 | 38.54 | 8.98 | 0.93 | 6.65 | 40.54 | 16.52 | 34.06 | 13.97 | 0.84 |
| 10 | 12.47 | 43.26 | 13.95 | 36.38 | 7.88 | 0.84 | 12.47 | 41.76 | 16.89 | 31.19 | 11.67 | 0.75 |
| 11 | 13.62 | 43.43 | 14.75 | 34.87 | 7.57 | 0.80 | 13.62 | 41.54 | 17.22 | 30.31 | 11.72 | 0.73 |
| 12 | 6.06 | 42.37 | 12.73 | 37.24 | 8.21 | 0.88 | 6.06 | 39.91 | 15.68 | 33.96 | 13.34 | 0.85 |
| 13 | 7.84 | 42.41 | 13.05 | 37.82 | 8.45 | 0.89 | 7.84 | 41.24 | 16.15 | 32.93 | 13.55 | 0.80 |
| 14 | 7.76 | 42.34 | 13.08 | 37.94 | 7.79 | 0.90 | 7.76 | 41.00 | 16.34 | 32.95 | 12.72 | 0.80 |
| 15 | 7.13 | 42.85 | 14.10 | 37.06 | 7.74 | 0.86 | 7.13 | 40.63 | 16.67 | 33.68 | 13.91 | 0.83 |
| 17 | 18.09 | 43.37 | 14.08 | 34.91 | 7.74 | 0.80 | 18.09 | 42.62 | 17.25 | 29.77 | 9.58 | 0.70 |
| 18 | 1.65 | 39.88 | 8.93 | 39.56 | 10.31 | 0.99 | 1.65 | 37.03 | 11.79 | 36.10 | 14.79 | 0.97 |
| <i>r^b</i> | 1.00 | 0.90* | 0.87* | -0.97* | -0.87* | -0.96* | 1.00 | 0.87* | 0.83* | -0.98* | -0.97* | -0.94* |

^(a) Mean value of five experiments (n = 10).

^(b) Regression coefficient – (*) significant up to 1 % level.

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Table 5. Modifications of the rheological properties of wheat flours from ozonated grain of two wheat genotypes (Diamento and Rubisko).

| Run | Diamento | | | | | Rubisko | | | | |
|----------------------|---|--|---------------------|------------------------|------------------|---|--|---------------------|------------------------|------------------|
| | O ₃ cons. (g.kg ⁻¹ grain) | W ^a (10 ⁻⁴ J) | P ^a (mm) | L ^a (mm) | P/L ^a | O ₃ cons. (g.kg ⁻¹ grain) | W ^a (10 ⁻⁴ J) | P ^a (mm) | L ^a (mm) | P/L ^a |
| T | 0.00 | 169 | 89 | 47 | 1.89 | 0.00 | 114 | 55 | 66 | 0.83 |
| 1 | 2.16 | 135 | 97 | 31 | 3.13 | 1.91 | 109 | 59 | 52 | 1.13 |
| 2 | 4.01 | 135 | 99 | 31 | 3.19 | 3.91 | 103 | 52 | 55 | 0.95 |
| 3 | 4.97 | 104 | 96 | 24 | 4.00 | 5.40 | 106 | 61 | 45 | 1.36 |
| 4 | 2.57 | 158 | 104 | 34 | 3.06 | 2.86 | 100 | 58 | 49 | 1.18 |
| 5 | 2.79 | 134 | 123 | 24 | 5.12 | 3.12 | 120 | 73 | 45 | 1.62 |
| 6 | 4.84 | 105 | 105 | 23 | 4.57 | 5.18 | 108 | 61 | 45 | 1.36 |
| 7 | 11.12 | 92 | 92 | 24 | 3.83 | 11.50 | 88 | 76 | 26 | 2.92 |
| 8 | 6.73 | 126 | 124 | 24 | 5.17 | 6.38 | 107 | 73 | 38 | 1.92 |
| 9 | 6.28 | 95 | 104 | 21 | 4.95 | 6.65 | 104 | 83 | 29 | 2.86 |
| 10 | 12.28 | 76 | 90 | 20 | 4.50 | 12.47 | 76 | 80 | 23 | 3.48 |
| 11 | 14.51 | 92 | 99 | 23 | 4.30 | 13.62 | 99 | 97 | 23 | 4.22 |
| 12 | 6.49 | 109 | 119 | 21 | 5.67 | 6.06 | 91 | 77 | 28 | 2.75 |
| 13 | 7.87 | 109 | 105 | 27 | 3.89 | 7.84 | 126 | 74 | 44 | 1.68 |
| 14 | 7.97 | 108 | 107 | 24 | 4.46 | 7.76 | 120 | 87 | 33 | 2.64 |
| 15 | 7.54 | 80 | 74 | 25 | 2.96 | 7.13 | 108 | 77 | 33 | 2.33 |
| 17 | 16.11 | 63 | 80 | 19 | 4.21 | 18.09 | 58 | 69 | 21 | 3.29 |
| 18 | 1.42 | 153 | 104 | 33 | 3.15 | 1.65 | 115 | 67 | 49 | 1.37 |
| <i>r^b</i> | 1.00 | -0.866* | --- | --- | 0.605* | 1.00 | -0.718* | --- | --- | 0.856* |

^(a) Mean value of five experiments (n = 5).^(b) Regression coefficient – significant up to 5 % level.

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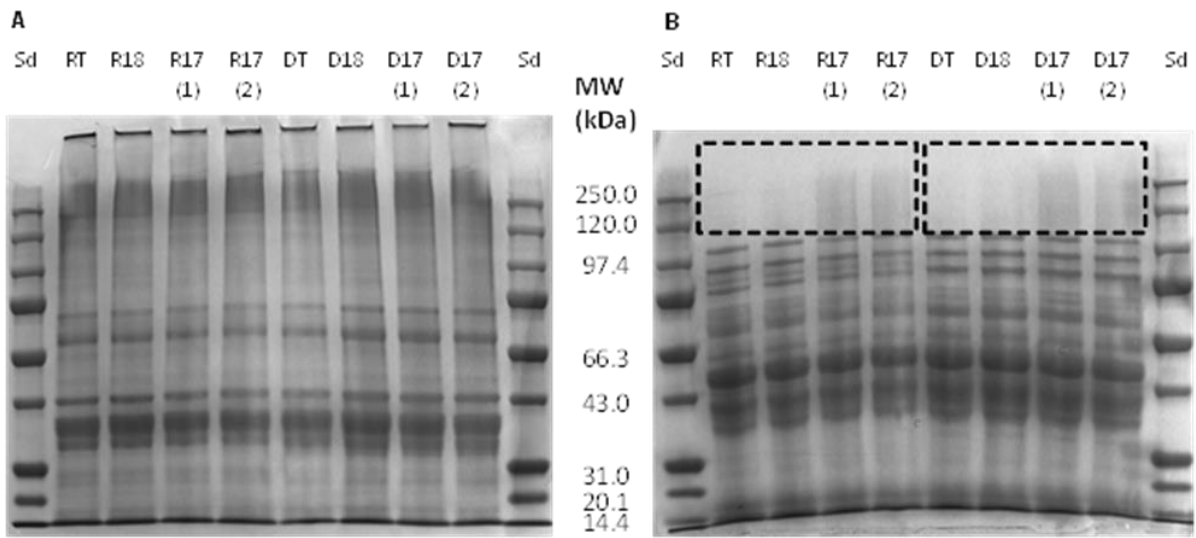
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630 Figure 1

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