



## Effects of ozone treatment on the molecular properties of wheat grain proteins

Perrine Gozé, Larbi Rhazi, Lyès Lakhal, Philippe Jacolot, André Pauss,  
Thierry Aussenac

### ► To cite this version:

Perrine Gozé, Larbi Rhazi, Lyès Lakhal, Philippe Jacolot, André Pauss, et al.. Effects of ozone treatment on the molecular properties of wheat grain proteins. *Journal of Cereal Science*, 2017, 75, pp.243-251. 10.1016/j.jcs.2017.04.016 . hal-03172583

**HAL Id: hal-03172583**

**<https://hal.science/hal-03172583>**

Submitted on 17 Mar 2021

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

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

Perrine Gozé<sup>a</sup>, Larbi Rhazi<sup>b</sup>, Lyès Lakhal<sup>b</sup>, Philippe Jacolot<sup>b</sup>, André Pauss<sup>c</sup>, and  
Thierry Aussenac<sup>b,\*</sup>

<sup>a</sup>PAULIC Minoteries, Moulin du Gouret, 56920 Saint Gérard, France.

<sup>b</sup>Institut Polytechnique UniLaSalle, UP Transformations & Agro-Ressources, 19 rue Pierre  
Waguet, 60026 Beauvais cedex, France.

<sup>c</sup>Sorbonne universités, Université de Technologie de Compiègne, ESCOM, EA4297 TIMR,  
Centre de recherche Royallieu - CS 60319 - 60203 Compiègne cedex, France.

\*Corresponding author

Tel: + 0033(0)344062500

Fax: + 0033(0)344062526

E-mail: [thierry.aussenac@unilasalle.fr](mailto:thierry.aussenac@unilasalle.fr)

**ABSTRACT**

Ozone is a powerful and highly reactive oxidizing agent, which has found increasing applications in the field of grain processing. However, in some cases, O<sub>3</sub> can potentially promote oxidation and/or degradation of the chemical constituents of grains. Experiments were carried out to evaluate the specific effects of gaseous ozone on the molecular properties of wheat grain proteins and their consequences on the bread-making quality of the resulting flours.

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

**Keywords:**

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

## 1. Introduction

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

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

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

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

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

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

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

## 2. Material and methods

### 2.1. Plant material

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

### 2.2. Ozone treatment

All experiments were conducted with a specific reactor set up at the Institut Polytechnique UniLaSalle. A 10 kg wheat grain mass was fed into a semi-batch stainless steel reactor equipped with a central endless screw for grain circulation. The temperature in the reactor was maintained at 22 °C by a cooling system. The reactor pressure was maintained at 0.3 bars by a gas vector. Ozone gas was fed into the bottom of the reactor through a micro-porous plate with a flow rate of 2.0 m<sup>3</sup> NTP.h<sup>-1</sup>. Ozone was produced from either dry air (dew point -70 °C) or oxygen (99.5 %, Air-liquid, France), which were connected to an ozone generator (OZAT<sup>®</sup> CFS-2G, Ozonia, France). Inlet and outlet ozone concentrations were continuously measured and recorded (Ozone Analyzer BMT 964). The ozone off gas was destroyed at 350 °C by a thermal destruction unit.

### 2.3. Experimental design

In order to generate a wide range of wheat grain samples differentiated in terms of ozone oxidation (i.e. amount of O<sub>3</sub> consumed), fifteen experiments with several combinations of (i) pre-humidification level (coded as  $X_1$ ) during preparation of the grain, (ii) ozone concentration

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

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

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

#### 2.5. *Alveographic measurements*

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

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

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

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

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

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

## 2.8. FT-IR analysis

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



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

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

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

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

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

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

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

#### *2.10. Determination of glutenin free sulfhydryl (SH) groups*

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

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

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

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

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

## **3. Results**

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

As can be seen in Table 2, the experimental design and the variables selected during this experiment (particularly pre-humidification level and ozone concentration in the ozone inlet) generated a wide range of oxidation of the samples; the latter was estimated on the basis of the total quantity of gaseous ozone consumed by the wheat grains during their processing. Thus, whatever the chemical composition of the plant material used (Diamento or Rubisko), the treatment rate of the grains obtained varied from 0.00 to 16.11 g O<sub>3</sub>.kg<sup>-1</sup> and 0.00 to 18.09 g O<sub>3</sub>.kg<sup>-1</sup> for Diamento and Rubisko, respectively.

The ozone treatment significantly modified the 2 % SDS solubility of flour proteins, which was evaluated by the UPP/EP ratio. For example, for the cultivar Rubisko, this ratio increased from 0.34 to 0.58 for the control and run17 (maximum O<sub>3</sub> consumption), respectively. The same observation was made with the cultivar Diamento (increase in UPP/EP ratio from 0.63 to 0.97 for the control and run17, respectively). Initial differences in the UPP/EP ratio between the two wheat genotypes (0.34 vs. 0.63 for Rubisko and Diamento, respectively) were due to the allelic variations coded in particular by the *Glu-1D* locus (Carceller and Aussenac, 2001). In fact, the presence of the 5 and 10 HMW-GS from Diamento decreased the initial 2 % SDS solubility of glutenins in contrast to the 2 and 12 HMW-GS from Rubisko.

At the same time, the ozone treatment significantly modified the MWD of flour proteins, which was mainly evaluated by the number-average molar mass ( $M_n$ ) and the weight-average molar mass ( $M_w$ ). Both  $M_n$  and  $M_w$  of the polymeric proteins significantly increased [from 9.38×10<sup>5</sup> g.mol<sup>-1</sup> and 3.81×10<sup>7</sup> g.mol<sup>-1</sup> to 15.34×10<sup>5</sup> g.mol<sup>-1</sup> and 6.77×10<sup>7</sup> g.mol<sup>-1</sup> for  $M_n$  and  $M_w$  of the control and run17 (maximum O<sub>3</sub> consumption), respectively] as a result of the grain ozonation. The same results were observed with the other hydrodynamic parameters, such as the number-average radii of gyration ( $R_n$ ) and the weight-average radii of gyration ( $R_w$ ).

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

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

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

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

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

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

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

### 3.3. *Protein secondary structure modifications*

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

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

#### 3.4. *Rheological properties of wheat flours*

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



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

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

#### **4. Discussion**

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

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

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

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

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

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

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

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

## **5. Conclusions**

As a result of this experimental work, it is clear that although the ozonation process is currently being used in the field of cereal processing mainly to meet food safety requirements, ozone may led to a modification of the majority of the techno-functional properties of processed products (i.e. flours and/or doughs). As our results show, these changes in techno-functional properties are mainly due to changes in the aggregate status of prolamins in grain. Given the action of ozone on the aggregative status of prolamins, the technological implementation of the flours generated requires specific and essential adjustments so that the professional can take full advantage of their new functionalities. Finally, the effect of gaseous O<sub>3</sub> on the other grain constituents (i.e. arabinoxylans and  $\beta$ -glucans) is currently being investigated.

## **6. Acknowledgement**

The authors would like to thank Dr Christian Coste for his kind help and suggestions  
and the ANRT (French National Agency for Research and Technology) for supporting part of  
this research.

**References**

- Alexandre, E.M.C., Brandão, T.R.S., Silva, C.L.M. (2011). Modelling Microbial Load Reduction in Foods Due to Ozone Impact. *Procedia Food Science*, 1, 836-841.
- Carceller, J.-L., Aussenac, T. (2001). Size Characterisation of Glutenin Polymers by HPSEC-MALLS. *Journal of Cereal Science*, 33, 131-142.
- Chittrakorn, S., Earls, D., MacRitchie, F. (2014). Ozonation as an alternative to chlorination for soft wheat flours. *Journal of Cereal Science*, 60, 217-221.
- Coste, C., Bailli, A., Dubois, M. (2005). Method for ozone treatment of plant materials. French Patent FR 2845015 (A1).
- Fleissner, G., Hage, W., Hallbrucker, A., Mayer, E. (1996). Improved curve resolution of highly overlapping bands by comparison of fourth-derivative curves. *Applied Spectroscopy*, 50, 1221-1337.
- Gozé, P., Rhazi, L., Pauss, A., Aussenac, T. (2016). Starch characterization after ozone treatment of wheat grains. *Journal of Cereal Science*, 70, 207-213.
- Gupta, R.B., Khan, K., MacRitchie, F. (1993). Biochemical basis of flour properties in bread wheats. I. Effects of variation in the quality and size distribution of polymeric protein. *Journal of Cereal Science*, 18, 23-41.
- Griffiths, P.R., Pariente, G.L. (1986). Introduction to spectral deconvolution. *Trends in Analytical Chemistry*, 5, 209-215.
- Hanft, F., Koehler, P. (2005). Quantitation of dityrosine in wheat flour and dough by liquid chromatography-tandem mass spectrometry. *Journal of Agriculture and Food Chemistry*, 53, 2418-2423.

- 495 Khadre, M.A., Yousef, A.E., Kim, J.G. (2001). Microbial aspects of ozone applications in food:  
496 a review. *Journal of Food Science*, 66, 1242-1252.
- 497 Klein, B., Levien Vanier, N., Moomand, K., Pinto, V.Z., Colussi, R., Da Rosa Zavareze, E.,  
498 Guerra Dias, A.R. (2014). Ozone oxidation of cassava starch in aqueous solution at  
499 different pH. *Food Chemistry*, 155, 167-173.
- 500 Lemelin, E., Aussenac, T., Violleau, F., Salvo, L., Lein, V. (2005). Impact of Cultivar and  
501 Environment on Size Characteristics of Wheat Proteins using Asymmetrical Flow Field-  
502 Flow Fractionation and Multi-Angle Laser Light Scattering. *Cereal Chemistry*, 82, 28-33.
- 503 Li, M., Zhu K.X., Wang B.W., Guo X.N., Peng, W., Zhou H.M. (2012). Evaluation of the  
504 quality characteristics of wheat flour and shelf-life of fresh noodles as affected by ozone  
505 treatment. *Food Chemistry*, 135, 2163-2169.
- 506 Linlaud, N., Ferrer, E., Puppo, M.C., Ferrero, C. (2011). Hydrocolloid interaction with water,  
507 protein, and starch in wheat dough. *Journal of Agricultural and Food Chemistry*, 59, 713-  
508 719.
- 509 Popineau, Y., Bonenfant, S., Cornec, M., Pezolet, M. (1994). A study by infrared spectroscopy  
510 of the conformations of gluten proteins differing in their gliadin and glutenin compositions.  
511 *Journal of Cereal Science*, 20, 15-22.
- 512 Rakita, S., Pojić, M., Torbica, A. (2014). Determination of free sulphydryl groups in wheat  
513 gluten under the influence of different time and temperature of incubation: Method  
514 validation. *Food Chemistry*, 150, 166-173.
- 515 Rodriguez-Mateos, A., Millar, S.J., Bhandari, D.G., Frazier R.A. (2006). Formation of  
516 dityrosine cross-links during breadmaking. *Journal of Agriculture and Food Chemistry*,  
517 54, 2761-2766.

- 518 Sandhu, H.P.S., Manthey, F.A., Simsek, S. (2011). Quality of bread made from ozonated wheat  
519 (*Triticum aestivum* L.) flour. *Journal of the Science of Food and Agriculture*, 91, 1576-1584.
- 520 Sandhu, H.P.S., Manthey, F.A., Simsek, S. (2012). Ozone gas affects physical and chemical  
521 properties of wheat (*Triticum aestivum* L.) starch. *Carbohydrate Polymers*, 87, 1261-1268.
- 522 Savi, G.D., Piacentini, K.C., Bittencourt, K.O., Scussel, V.M. (2014). Ozone treatment  
523 efficiency on *Fusarium graminearum* and deoxynivalenol degradation and its effects on  
524 whole wheat grains (*Triticum aestivum* L.) quality and germination. *Journal of Stored  
525 Products Research*, 59, 245-253.
- 526 Schofield, J.D., Booth, M.R. (1983). Wheat proteins and their technological significance. In  
527 B.J.F. Hudson, (Ed.), *Developments in Food Proteins* Vol. 2 (pp. 1-65). Barking, Essex, UK:  
528 Applied Science Publishers Ltd.
- 529 Shewry, P.R., Tatham, A.S. (1990). The prolamin storage proteins of cereal seeds: structure  
530 and evolution. *Biochemistry Journal*, 167, 1-12.
- 531 Southan, M., MacRitchie, F. (1999). Molecular weight distribution of wheat proteins. *Cereal  
532 Chemistry*, 76, 827-836.
- 533 Tiwari, B.K., Brennan, C.S., Curran, T., Gallagher, E., Cullen, P.J., O' Donnell, C.P. (2010).  
534 Review: Application of ozone in grain processing. *Journal of Cereal Science*, 51, 248-255.
- 535 Trombete, F.M., Freitas-Silva, O., Saldanha, T., Venâncio, A.A., Fraga, M.E. (2016). Ozone  
536 against mycotoxins and pesticide residues in food: Current applications and perspectives.  
537 *International Food Research Journal*, 23, 2545-2556.
- 538 Verweij, H., Christianse, K., Vansteveninck, J. (1982). Ozone-induced formation of O,O'-  
539 Dityrosine cross-links in proteins. *Biochimica et Biophysica Acta*, 70, 180-184.



Violleau, F., Pernot, A.-G., Surel, O. (2012). Effect of Oxygreen wheat ozonation process on bread dough quality and protein solubility. *Journal of Cereal Science*, 55, 115-124.

Weegels, P.L., Hamer, R.J., Schofield, J.D. (1996). Critical review: Functional properties of wheat glutenin. *Journal of Cereal Science*, 23, 1-18.

Wu, J., Doan, H., Cuenca, M.A. (2006). Investigation of gaseous ozone as an antifungal fumigant for stored wheat. *Journal of Chemical Technology and Biotechnology*, 81, 1288-1293.

Yvin, J.C., Bailli, A., Joubert, J.M., Bertaud, O. (2001). Method and installation for making flour from ozone-treated grains. French patent FR2802390 (A1).

**Figure captions**

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

588

**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      |
|             | ( $X_1$ )<br>[H <sub>2</sub> O % (v/w)] | ( $X_2$ )<br>(g.m <sup>-3</sup> NTP) | ( $X_3$ )<br>(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 ( $X_1$ )<br>[H <sub>2</sub> O % (v/w)] | Inlet ozone concentration ( $X_2$ )<br>(g.m <sup>-3</sup> NTP) | Reaction time ( $X_3$ )<br>(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 <sup>a</sup> | 1  | 1  | 1                                |
| 18 <sup>a</sup> | -1   | -1   | -1                               |

<sup>(a)</sup> Extra runs.

589

590

591

592

593

594

595

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

| Run                  | Diamento   |                     |  |  |                                     |                                     |                |   |
|----------------------|--|---------------------|--|--|-------------------------------------|-------------------------------------|----------------|---|
|                      | O <sub>3</sub> cons.<br>(g.kg <sup>-1</sup> grain) | UPP/EP <sup>a</sup> | M <sub>w</sub> <sup>a</sup> (×10 <sup>7</sup><br>g.mol <sup>-1</sup> ) | M <sub>n</sub> <sup>a</sup> (×10 <sup>5</sup><br>g.mol <sup>-1</sup> ) | R <sub>n</sub> <sup>a</sup><br>(nm) | R <sub>w</sub> <sup>a</sup><br>(nm) | υ <sup>b</sup> | [free SH] <sup>a</sup><br>(μmol.g <sup>-1</sup> 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<sup>c</sup></i> | <b>1.00</b>  | <b>0.625*</b>       | <b>0.863*</b>  | <b>0.961*</b>  | <b>0.860*</b>                       | <b>0.934*</b>                       | <b>---</b>     | <b>-0.764*</b>  |

| Run                  | Rubisko  |                     |  |  |                                     |                                     |                |   |
|----------------------|--|---------------------|--|--|-------------------------------------|-------------------------------------|----------------|---|
|                      | O <sub>3</sub> cons.<br>(g.kg <sup>-1</sup> grain) | UPP/EP <sup>a</sup> | M <sub>w</sub> <sup>a</sup> (×10 <sup>7</sup><br>g.mol <sup>-1</sup> ) | M <sub>n</sub> <sup>a</sup> (×10 <sup>5</sup><br>g.mol <sup>-1</sup> ) | R <sub>n</sub> <sup>a</sup><br>(nm) | R <sub>w</sub> <sup>a</sup><br>(nm) | υ <sup>b</sup> | [free SH] <sup>a</sup><br>(μmol.g <sup>-1</sup> 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<sup>c</sup></i> | <b>1.00</b>  | <b>0.850*</b>       | <b>0.720*</b>  | <b>0.776*</b>  | <b>0.698*</b>                       | <b>0.578*</b>                       | <b>---</b>     | <b>-0.735*</b>  |

<sup>(a)</sup> Mean value of three experiments (n = 3); <sup>(b)</sup> Conformation plot slope; <sup>(c)</sup> Regression coefficient – (\*) significant up to 1 % level.

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

| Run | Diamento   |                 |  |                 | Rubisko  |                 |  |                 |
|-----|--|-----------------|--|-----------------|--|-----------------|--|-----------------|
|     | Dityrosine<br>( $\mu\text{g.g}^{-1}\text{flour}$ ) |                 | Dityrosine<br>( $\text{nmol.g}^{-1}\text{flour}$ ) |                 | Dityrosine<br>( $\mu\text{g.g}^{-1}\text{flour}$ ) |                 | Dityrosine<br>( $\text{nmol.g}^{-1}\text{flour}$ ) |                 |
|     | Mean <sup>a</sup>                                  | SD <sup>b</sup> | Mean <sup>a</sup>                                  | SD <sup>b</sup> | Mean <sup>a</sup>                                  | SD <sup>b</sup> | Mean <sup>a</sup>                                  | SD <sup>b</sup> |
| 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           |

<sup>(a)</sup> Mean value of five experiments (n = 3).

<sup>(b)</sup> Standard Deviation.

607

**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 <sub>3</sub> cons.<br>(g.kg <sup>-1</sup><br>grain) | β-sheet <sup>a</sup><br>(%) | Random coil <sup>a</sup><br>(%) | α-helix <sup>a</sup><br>(%) | β-turn <sup>a</sup><br>(%) | α-helix/<br>β-sheet | O <sub>3</sub> cons.<br>(g.kg <sup>-1</sup><br>grain) | β-sheet <sup>a</sup><br>(%) | Random coil <sup>a</sup><br>(%) | α-helix <sup>a</sup><br>(%) | β-turn <sup>a</sup><br>(%) | α-helix/<br>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<sup>b</sup></i> | <b>1.00</b>   | <b>0.90*</b>                | <b>0.87*</b>                    | <b>-0.97*</b>               | <b>-0.87*</b>              | <b>-0.96*</b>       | <b>1.00</b>   | <b>0.87*</b>                | <b>0.83*</b>                    | <b>-0.98*</b>               | <b>-0.97*</b>              | <b>-0.94*</b>       |

(<sup>a</sup>) Mean value of five experiments (n = 10).  
(<sup>b</sup>) Regression coefficient – (\*) significant up to 1 % level.

608

609

610

611

612

613

614

615

616

617

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

| Run                  | Diamento  |  |                     |                        |                  | Rubisko   |  |                     |                        |                  |
|----------------------|---|--|---------------------|------------------------|------------------|---|--|---------------------|------------------------|------------------|
|                      | O <sub>3</sub> cons.<br>(g.kg <sup>-1</sup><br>grain) | W <sup>a</sup><br>(10 <sup>-4</sup> J) | P <sup>a</sup> (mm) | L <sup>a</sup><br>(mm) | P/L <sup>a</sup> | O <sub>3</sub> cons.<br>(g.kg <sup>-1</sup><br>grain) | W <sup>a</sup><br>(10 <sup>-4</sup> J) | P <sup>a</sup> (mm) | L <sup>a</sup><br>(mm) | P/L <sup>a</sup> |
| 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<sup>b</sup></i> | <b>1.00</b>   | <b>-0.866*</b>                         | <b>---</b>          | <b>---</b>             | <b>0.605*</b>    | <b>1.00</b>   | <b>-0.718*</b>                         | <b>---</b>          | <b>---</b>             | <b>0.856*</b>    |

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

618

619

620

621

622

623

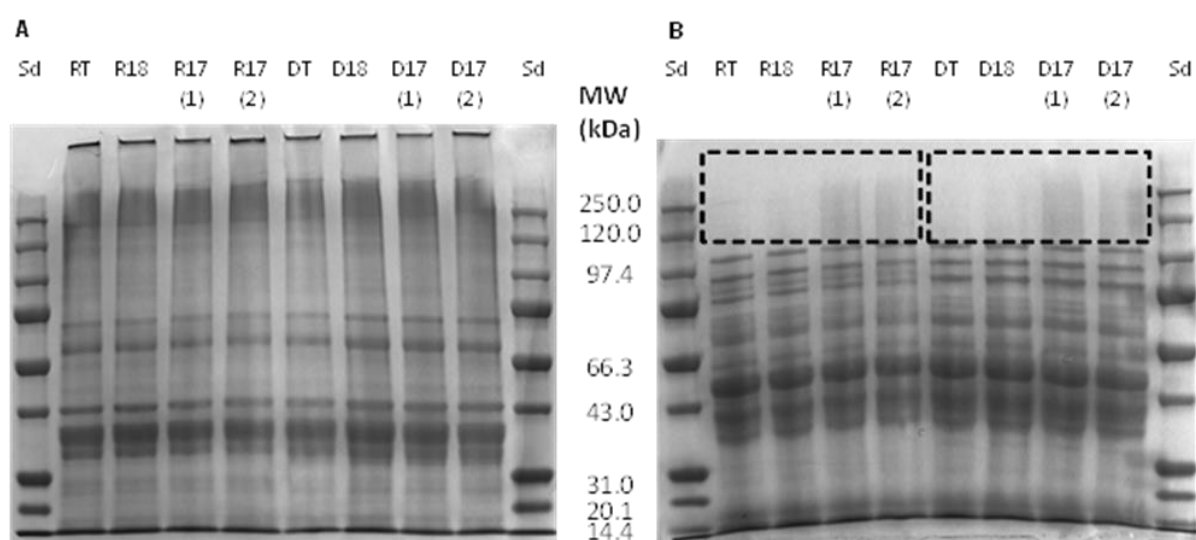
624

625

626

627

628



629

630 Figure 1

631

632

633