

How microwave treatment of gluten affects its toxicity for celiac patients? A study on the effect of microwaves on the structure, conformation, functionality and immunogenicity of gluten

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ABSTRACT

The microwave heating of wheat kernels, flour, and gluten, has attracted attention lately because it has been claimed to abolish gluten toxicity for celiac patients. Nevertheless, contradictory results have been reported regarding the effect on gluten celiac-immunotoxicity. In order to better understand the effect of the microwave treatment on gluten structure, conformation, functionality and celiac-immunotoxicity, a central composite design with two factors, power level, and treatment time, was used to investigate a possible quadratic and interaction effects between both factors. Extractable gliadins content was affected by the power and time in a linear and quadratic fashion; extractable glutenins were not affected. Gluten secondary structure was affected by the microwave treatment and related to the polymer's disaggregation phenomenon observed. In fact, the microwave treatment increased the amount of potentially toxic epitopes released after peptic and tryptic digestion, showing inefficiency as a treatment to detoxify the gluten for celiac disease patients.

1. Introduction

Celiac disease (CD) is a complex disorder characterized by an immune-mediated reaction to gluten that occurs in genetically predisposed individuals. Celiac patients react to dietary proteins, named prolamins, which are rich in proline and glutamine residues, including wheat gliadins and glutenins, rye secalins and barley hordeins (Lebwohl, Sanders, & Green, 2018). Statistics indicate that approximately 1% of the global population suffers from CD. In some countries like Algeria, the prevalence of CD is even higher reaching approximately 5.6% (Tonutti & Bizzaro, 2014). Its prevalence continues to be on the rise, and patients suffering from CD are faced with the daunting task of completely eliminating gluten from their diets in order to ameliorate or normalize the symptomatology, as this is the only treatment available. This task is made even more burdensome by the high

cost of gluten-free foods. Also, their technological performances are barely satisfactory (Zannini, Kingston, Arendt, & Waters, 2013). Moreover, the gluten-free diet can cause nutrient deficiencies because gluten-free products often contain less fibre, magnesium, and folic acid than gluten products and higher amounts of saturated fat, total fat and sugar (Vici, Belli, Biondi, & Polzonetti, 2016).

In this context, some research studies are being conducted aimed at the improvement of the technological, nutritional and organoleptic properties of gluten-free foods (Bourekoua, Benatallah, Zidoune, & Rosell, 2016; Capriles, dos Santos, & Areas, 2016). Other studies are focused on the chemical and enzymatic modification of gluten in order to reduce its immunological and toxic properties, in particular, the transamidation of glutamine residues (Gianfrani et al., 2007; Ribeiro et al., 2015) or the proteolysis of immunodominant epitopes (Pyle et al., 2005). The molecular reorganization of gluten to a new supramolecular

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architecture induced by chitosan was also recently proposed (Ribeiro et al., 2018). Physical treatments, like the microwave treatment of wheat kernels, were proposed to abolish the immunogenic properties of gluten proteins for celiac patients (Lamacchia, Landriscina, & D'Agnello, 2016), nevertheless the use of the microwave treatment for abolishing wheat gluten toxicity remains controversial as there is scientific evidence showing the inefficiency of this approach (Gianfrani et al., 2017; Leszczynska, Łacka, Szmraj, Lukamowicz, & Zegota, 2003). On the other hand, the microwave treatment of wheat flour and gluten results in a change of its rheological properties and functionality depending on the power and treatment time applied. A decrease in the bread-making potential is often observed with increasing time of exposure to the microwaves (Qu, Wang, Liu, Wang, & Liu, 2017). Moreover, the microwave treatment has been mostly applied to wheat kernels and flours being difficult to isolate the effect on gluten proteins only. Therefore, the aim of the present study was to gain a deeper understanding of the effect of the microwave treatment of gluten on its functionality, structure, conformation and more importantly on the gluten celiac-related toxicity. For this purpose, a central composite design was used in order to explore the combined effect of power and treatment time or their interaction on gluten properties aiming to build a deeper understanding of the microwave treatment of gluten and its potential for the development of an alternative to the gluten-exclusion diet.

2. Materials and methods

2.1. Materials

Fine powder of vital wheat gluten was provided by PPlus Ingredients Company (Algiers, Algeria) with the following composition according to the manufacturer: 80% of protein, 6% of moisture, 1% of ash, 7% of total fat, 9% of starch and less than 1% of cellulose.

2.2. Experimental design

In order to evaluate the linear and quadratic effects of the microwave power level (X1) and time of microwave treatment (X2) and their interaction (X1X2) on the gluten properties under study (temperature, moisture content, extractable gliadins, extractable glutenins, free sulfhydryl group content, R5 reactivity of the peptides released after simulated gastric and intestinal digestion, maximum resistance to extension - R_{max} and total extensibility - Ext) a response surface methodology (RSM) was carried out using a central composite design (CCD). The SDS-PAGE profiles, the secondary structure and the macromolecular features of the polymers were studied independently to the RSM. The ranges of the variables were selected according to preliminary tests conducted by heating the gluten (minimum values) and avoid burning it (maximum values). The microwave treatment was performed with domestic microwave equipment (Condor, Algeria) and the values of two variables, power level, and treatment time, are indicated in Supplementary Table 1. Thirteen experiments were performed: four tests of factorial points, four axial points and five repetitions of the central point (Supplementary Table 2). The effects of the two factors on the different responses were represented using the following polynomial quadratic regression equation:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_{11}X_1^2 + b_{22}X_2^2 + b_{12}X_1X_2$$

where:

- Y: response variable;
- b_0 : intercept;
- b_1 and b_2 : linear regression coefficients;
- b_{11} b_{22} : quadratic regression coefficients;
- b_{12} : Interaction regression coefficient.

Regression coefficients were calculated with normalized factors and using pure error as the ANOVA error term. The non-significant effects were ignored for the graphical representation.

2.3. Temperature monitoring

The temperature of the gluten samples treated by the microwaves was monitored using an electronic probe thermometer. The temperature was measured in duplicate.

2.4. Determination of moisture content

The moisture content of the control gluten and the microwave treated glutes, was determined according to the AACC Approved Method 44-19 (AACC, 1999). The moisture content was measured in duplicate.

2.5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE experiments were performed using the sequential procedure of Singh, Shepherd, and Cornish (1991). Gliadins and glutenins of gluten were extracted from a fine powder of wheat gluten. Briefly, gliadins were extracted with 50% (v/v) aqueous 1-propanol, dried at 65 °C overnight and dissolved in the sample buffer. The pellet containing the glutenins was washed twice with 50% (v/v) aqueous 1-propanol to ensure complete removal of gliadins. Then, the glutenins were reduced and alkylated in a 50% (v/v) 1-propanol solution with 1% (w/v) dithiothreitol (DTT) and 1.4% (w/v) 4-vinylpyridine, respectively. After centrifugation, the sample buffer (1:1) was added to the supernatant. The gliadin and glutenin fractions were separated in a resolving gel using 10.3% and 12.5% T (total monomer percentage); 1.3% and 0.97% C (weight percentage of crosslinker), respectively, in a vertical electrophoresis system (Hoefer, SE600, San Francisco, CA, USA).

2.6. Quantification of gluten proteins by reversed phase-high performance liquid chromatography (RP-HPLC)

The gliadins and glutenins were separated according to their hydrophobicity by RP-HPLC as described by Wieser, Antes, and Seilmeier (1998) after extraction using the method described above (Singh et al., 1991). For the HPLC analysis, a RP-C8 column was used (Macherey-Nagel, Germany, 25 cm × 4.5 mm i.d. × 5 μm) and maintained at 50 °C during the separation process. An injection volume of 50 μL for the glutenins and 100 μL for the gliadins was used. A gradient elution was performed using the following eluents: eluent A – 0.1% (v/v) aqueous trifluoroacetic acid and eluent B - acetonitrile and trifluoroacetic acid (99.9/0.1%, v/v). The following elution program was used: 0 min 28% B, 30 min 56% B, using a flow rate of 1 mL/min. Detection was performed by ultraviolet (UV) absorbance at 210 nm. After each analysis, the column was cleaned by using 90% B during 5 min and equilibrated to 28% B during 10 min. The quantification of proteins was performed using a standard curve of bovine serum albumin (Ribeiro et al., 2016).

2.7. Free sulfhydryl content analysis

The free sulfhydryl content of gluten was determined according to the method of Chan and Wasserman (Chan & Wasserman, 1993) with slight modifications (Tuhumury, Small, & Day, 2014). Gluten samples (10 mg) were suspended in 1.0 mL of reaction buffer containing 8 mol/L urea, 3 mmol/L EDTA, 1% SDS, and 0.2 mol/L Tris-HCl, pH 8.0 (Buffer A). Samples were vortexed for 30 s and shaken on a constant platform shaker at room temperature for 1 h. Buffer B containing 10 mmol/L 5,5'-Dithiobis-2-nitrobenzoic acid (DTNB) in 0.2 mol/L Tris-HCl, pH 8.0

(0.1 mL) was then added to each sample and shaken for a further period of 1 h. Samples were then centrifuged at 13,600g for 15 min at room temperature. The absorbance of the supernatant was determined at 412 nm using an UV-VIS spectrophotometer (LLG-uniSPEC 2, France) against a blank containing 1.0 mL of buffer A and 0.1 mL of buffer B (Tuhumury et al., 2014). The concentration of free sulfhydryl groups expressed as ($\mu\text{mol/g}$ of protein) was calculated using the following equation:

$$\text{Free SH} = \frac{A \times D}{\epsilon \times C}$$

Where:

- A: absorbance at 412 nm;
- D: dilution factor;
- ϵ : extinction coefficient $13,600 \text{ M}^{-1} \text{ cm}^{-1}$;
- C: gluten concentration (mg/ml).

The analysis was performed in triplicate.

2.8. Changes in secondary structure of gluten proteins

To evaluate the changes in the secondary structure of gluten proteins when treated by microwaves, Fourier transform infrared spectroscopy (FTIR) was used. The FTIR spectra were recorded in the 4000 and 400 cm^{-1} range by co-addition of 128 scans and a 4 cm^{-1} resolution using an IRAffinity-1S Fourier Transform Infrared Spectrometer (Shimadzu, Izasa Scientific, France) equipped with a single-reflection diamond attenuated total reflection (ATR) crystal and a mercury-cadmium-telluride (MCT) detector. The measurement of each sample was repeated five times and averaged. The diamond crystal was carefully cleaned with 70% ethanol and distilled water after each measurement. The spectra collection and baseline correction were performed using the Lab solutions IR software. The number and the position of peaks were determined by the second derivative method. For deconvolution of the amide I band ($1600\text{--}1700 \text{ cm}^{-1}$) region the Peak fit software (v4.6, SPSS Inc, USA) was used. The iterative data fitting was performed until a correlation coefficient higher than 0.995 was obtained. The relative peak areas of the absorbance bands were expressed as a percentage of the area of the fitted region (Sivam, Sun-Waterhouse, Perera, & Waterhouse, 2012).

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

Samples were prepared and analysed according to the protocol of protein extraction, described in detail by (Lemelin, Aussenac, Violleau, Salvo, & Lein, 2005) and (Gozé et al., 2017). Briefly, flour samples (30 mg) were dispersed and incubated at 60°C for 15 min with 1.0 mL of 0.05 mol/L sodium phosphate buffer (pH 6.9) containing 2% (w/v) SDS. The extracts were then sonicated for 20 s 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 μL) into the AFFFF/MALLS system. Protein content was determined for each extract by combustion nitrogen analysis. 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), 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 μm . 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 mol/L, pH 6.9) containing 0.1% (w/v) SDS was used as mobile phase and was filtered through a $0.1 \mu\text{m}$ 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, followed by a focus/injection time of 1.0 min at 0.2 mL/min and a relaxation/focusing time of 0.5 min. Elution then followed at an outflow rate (F_{out}) of 1.0 mL/min and with a cross-flow rate (F_c) decreasing exponentially from 3.0 to 0.0 mL/min for 14 min. Finally, elution at a cross-flow rate of 0.0 mL/min was maintained for 9 min.

2.10. Size-exclusion chromatography (SEC)

Proteins from the control and microwave treated samples were extracted according to the SDS (sodium dodecyl sulphate) sequential extraction procedure (Carceller & Aussenac, 1999; Gupta, Khan, & Macritchie, 1993). The total polymeric protein was separated into two size fractions based on its extractability in 2% (w/v) SDS-phosphate buffer (without sonication), i.e. extractable ($F1s + F2s$) and unextractable ($F1i + F2i$) fractions. Briefly, samples (0.01 g) were suspended in 1 mL of 0.05 mol/L sodium phosphate buffer (pH 6.9) containing 2% (w/v) SDS and stirred for 2 h at 60°C . These extractions were followed by centrifugation (12,500g, 30 min at 20°C) to obtain a supernatant containing extractable polymeric protein EPP ($F1s + F2s$). Unextractable polymeric protein UPP ($F1i + F2i$) was then sonicated for 20 s at 30% power (output 25 W, 23 kHz) in 1 mL of 2% SDS-phosphate buffer using a stepped microtip probe (3 mm diameter) to solubilize the remaining protein (unextractable proteins). Both extracts were filtered through $0.45 \mu\text{m}$ regenerated cellulose filters (Gelman Sciences, France) and 25 μL were then injected onto a TSKgel G4000 SW (TOSOHAAS, Stuttgart, Germany) size exclusion analytical column ($7.5 \times 300 \text{ mm}$, $8 \mu\text{m}$). The HPLC apparatus was Agilent 1200 (Agilent Technologies, Germany) consisting of an online degasser, an isocratic pump, a variable auto-injector and a variable wavelength detector (set at 214 nm). Sodium phosphate buffer (0.1 mol/L, pH 6.9) containing 0.1% (w/v) SDS was used as eluent with a flow rate of 0.7 mL min^{-1} . During the fractionation, the column was maintained at 25°C . For the extractable fraction, the elution profile was divided into four main peaks corresponding to polymeric glutenin proteins ($F1s + F2s$), gliadins ($F3$) and functional proteins ($F4$) (Fig. 3C; Supplementary Fig. 1). The percentage of unextractable polymeric protein in total polymeric protein (TPP) was calculated as follows:

$$\begin{aligned} \frac{\text{UPP}}{\text{TPP}} (\%) &= \frac{\text{F1i} + \text{F2i areas (unextractable)}}{\text{F1s} + \text{F2s areas (extractable)} + \text{F1i} + \text{F2i areas (unextractable)}} \\ &\times 100 \end{aligned}$$

The unextractable polymeric protein to extractable polymeric protein ratio:

$$\frac{\text{UPP}}{\text{EPP}} = \frac{\text{F1i} + \text{F2i areas (unextractable)}}{\text{F1s} + \text{F2s areas (extractable)}}$$

The unextractable gliadin protein (Glii) to extractable gliadin protein (Glis) ratio:

$$\frac{\text{Glii}}{\text{Glis}} = \frac{\text{F3i areas (unextractable)}}{\text{F3s areas (extractable)}}$$

2.11. Micro-extension tests

Micro-extension tests on the rehydrated gluten were made using a texture analyser TA-XT2 (Stable Micro Systems, Godalming, Surrey, United Kingdom) fitted with the SMS/Kieffer dough and gluten

extensibility rig. First, 5 g of dried gluten were rehydrated with 10 mL of distilled water at 20 °C for 15 min and centrifuged (10 min, 5000g, 20 °C) (Kieffer, Schurer, Kohler, & Wieser, 2007). After a resting time of 20 min at 30 °C under a water-saturated atmosphere, wet gluten was brushed with paraffin oil, to avoid sample adhesion, and pressed into a Teflon mould pre-warmed to 30 °C. After a further 40 min at 30 °C under a water-saturated atmosphere, it was analysed. The maximum resistance to extension ($R_{\max}$) and total extensibility (Ext) were determined in triplicate (Ribeiro et al., 2015).

2.12. *In vitro* digestion and R5 competitive ELISA immunoassay

The R5 Competitive ELISA was used to quantify the potential immunogenic peptides (RIDASCREEN® Gliadin competitive, R-Biopharm AG Darmstadt, Germany). *In vitro* digestion of gluten was conducted as previously described (Gessendorfer, Koehler, & Wieser, 2009). First, control gluten and microwave treated gluten were dispersed in distilled water at a ratio of 5% (w/v) and the pH was adjusted to 1.8 using a solution of 1 mol/L HCl. After dispersion, 2.5 mg of pepsin (Sigma-Aldrich, Steinheim, Italy) were added and allowed to catalyse the gluten hydrolysis for 4 h under shaking at 37 °C. Subsequently, the pH was adjusted to 7.8 with NaOH 1 mol/L and 2.5 mg of trypsin (Gibco, Life Technology, UK) were added and the mixture was incubated as described above. Finally, the pH was adjusted to 4.5 with a solution of 1 mol/L HCl and the peptic-tryptic digests were centrifuged at 4000g for 20 min at room temperature. Then, the supernatant was freeze-dried. The peptic-tryptic digests (PT) were analysed in duplicate using the R5 Competitive ELISA immunoassay, using a microplate reader (Multiskan EX Labsystems, USA).

2.13. Statistical analysis

The results are expressed as mean $\pm$ standard deviation (SD). All results were corrected for a 14% moisture content. The experimental design and data analysis were performed using the statistical package STATISTICA from Statsoft (v10; Tulsa, Oklahoma, USA). The differences among the different means were determined by one-way analysis of variance (ANOVA). Multiple comparisons were performed using the two-stage step-up method of Benjamini, Krieger, and Yekutieli (2006).

3. Results and discussion

The gluten used in this work presented a protein profile (Ribeiro et al., 2015), polymeric composition and organization (unextractable and extractable polymeric protein) (Gupta et al., 1993) and hydrodynamic parameters of the polymeric material (Ribeiro et al., 2019) similar to those obtained for wheat flours, therefore being similar and representative of that present in unprocessed wheat flours.

The different parameters analysed, including the maximum temperature achieved during microwave treatment, moisture content, and content of gluten proteins measured by RP-HPLC, free SH groups, R5 reactivity after peptic and tryptic digestion, $R_{\max}$ and Ext, the abundance of secondary structures and the macromolecular features of polymers are shown in Table 1.

The regression coefficients for the significant effects of the microwave treatment parameters, power (X1) and time (X2), are presented in Table 2. The determination coefficient R^2 and significance ($p < 0.05$) indicated a good fitting of the model for the maximum temperature of the gluten achieved during the treatment, moisture content, extractable gliadins, $R_{\max}$ and Ext (Table 2).

3.1. Effect of microwave treatment on gluten proteins extractability and primary structure

The different conditions of the microwave treatment of gluten showed a different impact on the maximum temperature achieved. The

temperature ranged from 41.85 °C for gluten 5 to 77.80 °C for gluten 6. The increased agitation of the water molecules and charged ions, when exposed to the microwaves, is the basis for the conversion of the electromagnetic energy of the microwave to thermal energy. In addition, the microwave allows faster heating than the conventional heating methods, through the direct penetration of the microwaves (Tang & Resurreccion, 2009). For the power used, the linear and the quadratic effects were found significant. The treatment time also impacted the gluten temperature in a linear fashion, being also observed a significant interaction between treatment time and power (Fig. 1A). Overall, the temperature increased with increasing power and with increasing treatment time as shown in Fig. 1A, but the increase in temperature with treatment time is more pronounced for the higher power levels applied.

The gluten moisture content was only linearly and significantly affected by the power of the microwave treatment, decreasing with increasing power level (Fig. 1B). The moisture content of the microwave treated glutes varied from 6.5 to 7.5% whereas the moisture content of the control gluten was $8.29 \pm 0.06\%$. In fact, the moisture content of all the microwave-treated samples decreased and a significant negative correlation was found between temperature and moisture content ($r = -0.7535$, $p = 0.0029$), showing that, as expected, the heat generated by the microwave radiation resulted in the dehydration of gluten. Similar results were found by Diraman (2010).

Both power level and treatment time significantly influenced the content of extractable gliadins, showing significant linear and quadratic effects without significant interaction between them (Fig. 1C). The maximum content of extractable gliadins measured by RP-HPLC was achieved using the conditions of power level and treatment time near the maximum values used. In fact, the extractable gliadins obtained in these conditions were significantly higher than those observed for the control gluten (Table 1). On the other hand, the microwave treatment did not affect the content of extractable glutenins, i.e. the protein extracted after reduction with dithiothreitol (DTT). Furthermore, no significant differences were observed for the microwave treated glutes under different power levels and treatment times. In order to evaluate if the microwave treatment affected the primary structure of gluten proteins, glutenins and gliadins extracted from the control gluten and microwave treated glutes were separated according to their molecular weight by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Their electrophoretic profiles are shown in Fig. 2.

The SDS-PAGE results showed that both glutenins and gliadins from the microwave treated glutes did not present any significant difference in their electrophoretic profiles and also in relation to the control gluten, including any increase or decrease in the number of protein bands. The same migration pattern observed for all the samples indicates that the primary structure of the gluten proteins was not modified which in turn means that the microwave treatment did not induce gluten hydrolysis under the tested conditions. These results are in accordance with the literature where it is stated that microwaving cannot change the primary structure of proteins because the energy of the chemical bonds is far superior to the quantum energy of the microwave (Han, Cai, Cheng, & Sun, 2018). The fact that the extractability of gliadins has been significantly altered without a change in their primary structure suggests that changes in their secondary and tertiary structure are involved in this observed increase in extractability (further discussed below).

3.2. Effect of microwave treatment on gluten functionality, secondary structure and macromolecular features of polymers

Fig. 3 shows the effect of microwave treatment on the $R_{\max}$ (A) and Ext (B) of the glutes. $R_{\max}$ of the microwave treated glutes ranged from 64.63 ± 0.58 g to 142.17 ± 4.38 g (Table 1). When compared to the control gluten (91.47 ± 1.06 g) it can be observed that the microwave treatment decreased the $R_{\max}$ in some treatments, while

Table 1
General table gathering the experimental results. Differences in relation to the control are given in bold * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Gluten						
Parameter	1	2	3	4	5	6
Conditions	Control					
Power (W)	317.1	882.8	317.1	882.8	200	1000
Time (s)	0.00	25.85	25.85	54.14	54.14	40.00
Temperature (°C)	52.40 ± 0.56	63.20 ± 0.14	56.60 ± 0.56	75.80 ± 0.28	41.85 ± 0.91	77.80 ± 0.28
Moisture (g/100 g)	8.29 ± 0.06	7.67 ± 0.06***	7.74 ± 0.06***	5.97 ± 0.03***	7.63 ± 0.08***	6.76 ± 0.03***
Gluten proteins quantification	Glutenins (g/100 g)#	11.76 ± 0.79	11.28 ± 0.18	11.86 ± 1.05	11.53 ± 0.53	11.49 ± 0.23
	HMW-GS (g/100 g)#	2.71 ± 0.06	2.79 ± 0.19	2.74 ± 0.01	2.83 ± 0.15	2.75 ± 0.07
	LMW-GS (g/100 g)#	9.05 ± 0.39	10.58 ± 0.94***	8.54 ± 0.09	9.03 ± 0.46	8.74 ± 0.07
	Gliadins (g/100 g)#	49.97 ± 4.42	51.16 ± 3.16	49.62 ± 9.10	61.03 ± 3.77	60.15 ± 0.41
	ω5-gliadins (g/100 g)#	4.15 ± 0.17	3.45 ± 0.13***	3.49 ± 0.30***	4.16 ± 0.18	4.06 ± 0.18
	ω1.2-gliadins (g/100 g)#	4.34 ± 0.17	3.46 ± 0.11***	3.63 ± 0.34**	4.14 ± 0.01	4.33 ± 0.09
	α/β-gliadins (g/100 g)#	26.54 ± 1.15	24.57 ± 2.18	26.99 ± 0.29	33.83 ± 1.71***	32.99 ± 0.26***
Free SH (μmol/g)#	α/β-gliadins (g/100 g)#	14.93 ± 0.65	16.25 ± 0.71*	15.51 ± 0.17	18.90 ± 0.95***	18.77 ± 0.15***
	γ-gliadins (g/100 g)#	5.19 ± 0.12	4.87 ± 0.07	5.46 ± 0.04	5.59 ± 0.06*	5.34 ± 0.19
Rheology	R_{max} (g)	91.47 ± 1.06	137.75 ± 0.43***	64.63 ± 0.58***	142.17 ± 4.38***	103.25 ± 0.78**
	Ext (mm)	59.64 ± 2.05	62.77 ± 0.32	50.70 ± 0.44***	41.71 ± 0.33***	50.52 ± 2.62***
FTIR	1671 cm^{-1} (%)	0.719 ± 0.005	0.586 ± 0.005***	0.483 ± 0.002***	0.674 ± 0.006***	0.835 ± 0.002***
	1657 cm^{-1} (%)	0.978 ± 0.007	0.615 ± 0.002***	0.612 ± 0.003***	0.569 ± 0.005***	1.029 ± 0.002***
	1628 cm^{-1} (%)	1.061 ± 0.007	0.683 ± 0.006***	0.674 ± 0.003***	0.755 ± 0.007***	1.166 ± 0.003***
	1614 cm^{-1} (%)	0.506 ± 0.014	0.688 ± 0.012***	0.637 ± 0.007***	0.725 ± 0.008***	0.568 ± 0.005***
AF4	γ	0.42 ± 0.02	0.37 ± 0.04	0.33 ± 0.02***	0.36 ± 0.01	0.33 ± 0.02***
	Mn (kDa)	7979.50 ± 560.10	7500.43 ± 265.72	7445.13 ± 531.26	6788.80 ± 559.76	7758.70 ± 448.03
	Mw (kDa)	32862.23 ± 3044.02	30691.00 ± 1635.65	27536.10 ± 2029.01**	27705.30 ± 1535.58**	37460.15 ± 1269.25*
	M (Avg)	5117.30 ± 118.78	5154.87 ± 6.30	4392.90 ± 234.40	4681.53 ± 334.59	5120.57 ± 98.02
	Mw/Mn	4.11 ± 0.10	4.09 ± 0.09	4.63 ± 0.24*	4.10 ± 0.34	4.74 ± 0.05**
	Rw (nm)	65.77 ± 5.26	63.27 ± 3.33	61.13 ± 2.19	60.47 ± 1.27	62.37 ± 0.32
	Rz (nm)	100.33 ± 9.05	96.90 ± 7.34	95.20 ± 5.93	93.93 ± 5.73	95.40 ± 3.77
	R (Avg)	62.90 ± 2.34	65.93 ± 1.16	62.23 ± 1.37	66.07 ± 0.59	63.37 ± 1.62
	Rh (Q)	42.37 ± 1.19	42.57 ± 0.76	41.83 ± 1.42	42.90 ± 0.70	42.37 ± 0.38
	Polymers mass fraction (%)	16.99 ± 0.82	15.82 ± 0.93	10.84 ± 0.92***	18.18 ± 0.70	13.28 ± 1.43**
SEC	UPP/PPP	1.20 ± 0.18	0.81 ± 0.02***	0.69 ± 0.12***	0.65 ± 0.19***	0.65 ± 0.03***
	UPP/TPP (%)	54.26 ± 3.74	44.83 ± 0.57***	40.49 ± 4.2***	38.61 ± 7.17***	39.39 ± 1.12***
	GIII/GIIs	0.94 ± 0.13	0.59 ± 0.05***	0.46 ± 0.11***	0.34 ± 0.19***	0.39 ± 0.03***

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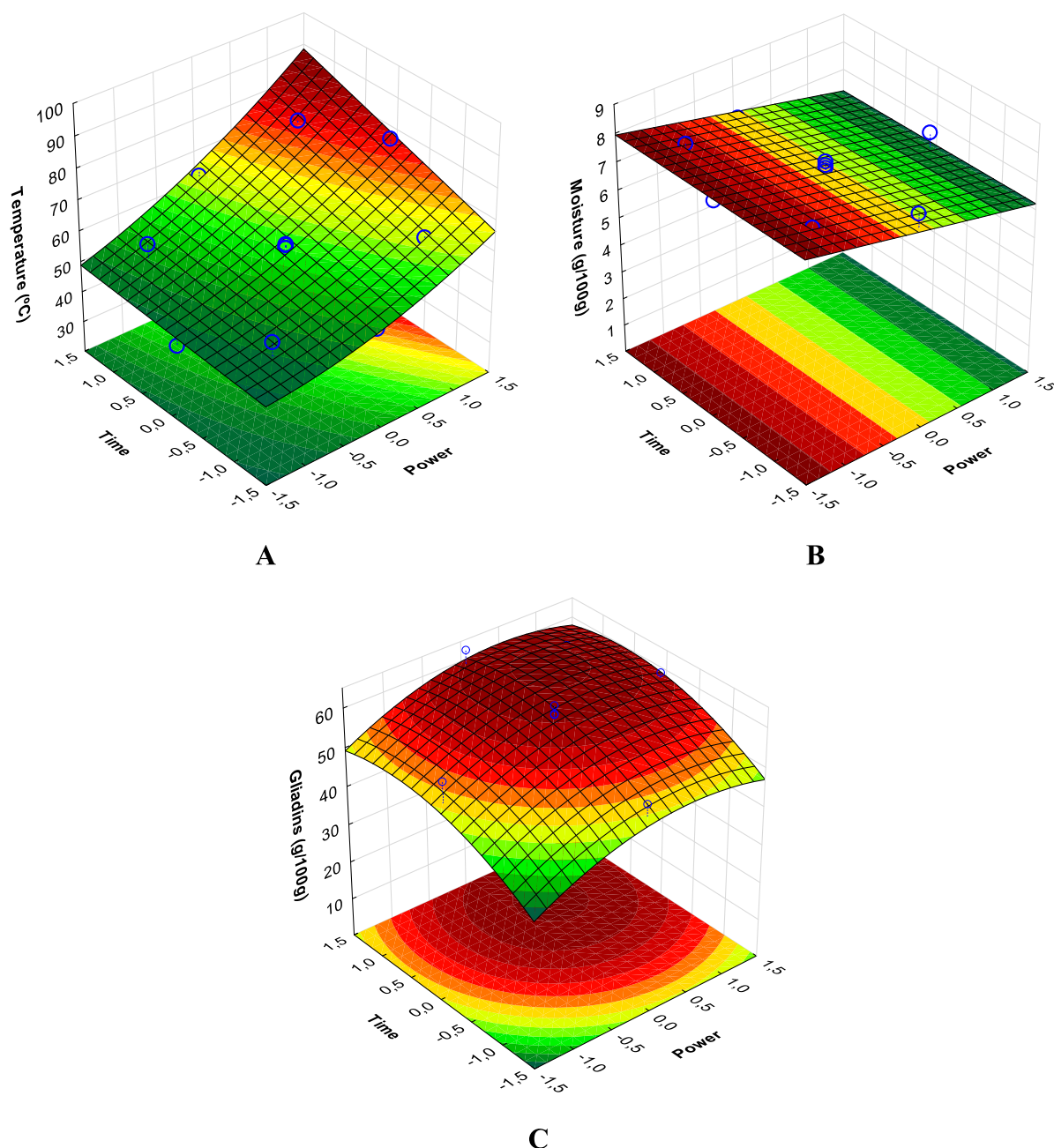
Table 1 (continued)

Parameter	Gluten											
	7	8	9	10	11	12	13					
Conditions	600	600.00	600	600	600	600	600					
Power (W)	40.00	20.00	60.00	40.00	40.00	40.00	40.00					
Time (s)	50.80 ± 1.13	63.00 ± 0.28	57.75 ± 0.63	57.65 ± 0.21	58.70 ± 0.14	58.45 ± 0.63	57.70 ± 0.56					
Temperature (°C)	7	8	9	10	11	12	13					
Moisture (g/100 g)	7.53 ± 0.07***	6.97 ± 0.03***	7.13 ± 0.08***	7.21 ± 0.03***	6.92 ± 0.08***	7.34 ± 0.07***	7.02 ± 0.04***					
Gluten proteins quantification												
Glutenins (g/100 g)#	12.90 ± 1.22	11.69 ± 0.98	11.49 ± 0.52	11.14 ± 0.35	13.43 ± 0.22	12.67 ± 0.24	10.88 ± 0.23					
HMW-GS (g/100 g)#	2.94 ± 0.09	2.80 ± 0.11	2.61 ± 0.02	2.64 ± 0.01	2.82 ± 0.09	2.91 ± 0.02	2.47 ± 0.03*					
LMW-GS (g/100 g)#	9.96 ± 0.59*	8.88 ± 0.46	8.87 ± 0.28	8.51 ± 0.19	10.60 ± 0.04***	9.77 ± 0.11*	8.41 ± 0.16					
Gliadins (g/100 g)#	52.85 ± 11.03	63.63 ± 1.55**	58.84 ± 2.04	63.11 ± 1.23**	58.95 ± 4.35	60.73 ± 8.55	60.87 ± 0.39					
ω5-gliadins (g/100 g)#	3.55 ± 0.47**	4.12 ± 0.02	3.96 ± 0.24	4.28 ± 0.03	4.04 ± 0.09	4.07 ± 0.42	4.28 ± 0.02					
ω1,2-gliadins (g/100 g)#	3.69 ± 0.53**	4.20 ± 0.03	4.12 ± 0.17	4.28 ± 0.12	4.55 ± 0.22	4.41 ± 0.46	4.67 ± 0.01					
α/β-gliadins (g/100 g)#	29.01 ± 1.72*	35.42 ± 1.82***	32.61 ± 1.02***	35.19 ± 0.80***	32.22 ± 0.14***	33.67 ± 0.39***	33.67 ± 0.63***					
γ-gliadins (g/100 g)#	16.61 ± 0.99**	19.89 ± 1.02***	18.15 ± 0.57***	19.36 ± 0.44***	18.14 ± 0.08***	18.58 ± 0.22***	18.25 ± 0.34***					
Free SH (μmol/g)#	5.36 ± 0.12	5.25 ± 0.06	5.12 ± 0.29	5.22 ± 0.24	5.42 ± 0.06	5.30 ± 0.07	5.33 ± 0.15					
Rheology												
R _{max} (g)	102.89 ± 0.55**	99.41 ± 4.71	99.05 ± 0.44	107.77 ± 13.15***	100.75 ± 1.87*	96.69 ± 6.00	98.79 ± 1.69					
Ext (mm)	45.12 ± 2.32***	43.23 ± 5.05***	57.65 ± 0.97	59.58 ± 0.78	59.72 ± 0.89	59.18 ± 0.89	59.56 ± 0.91					
FTIR												
1671 cm ⁻¹ (%)	0.906 ± 0.008***	0.631 ± 0.001***	0.666 ± 0.08***	0.666 ± 0.08***	0.849 ± 0.011***	0.849 ± 0.011***	0.849 ± 0.011***					
1657 cm ⁻¹ (%)	0.822 ± 0.007***	0.609 ± 0.001***	0.849 ± 0.011***	0.849 ± 0.011***	0.840 ± 0.011***	0.840 ± 0.011***	0.840 ± 0.011***					
1628 cm ⁻¹ (%)	1.021 ± 0.009***	0.754 ± 0.001***	0.840 ± 0.011***	0.840 ± 0.011***	0.840 ± 0.011***	0.840 ± 0.011***	0.840 ± 0.011***					
1614 cm ⁻¹ (%)	0.884 ± 0.011***	0.379 ± 0.003***	0.838 ± 0.017***	0.838 ± 0.017***	0.838 ± 0.017***	0.838 ± 0.017***	0.838 ± 0.017***					
AF4												
ν	0.35 ± 0.03	0.37 ± 0.01	0.33 ± 0.02***	0.38 ± 0.03	0.35 ± 0.03	0.34 ± 0.04**	0.36 ± 0.03					
Mn (kDa)	7793.53 ± 476.74	7042.90 ± 275.80	7495.93 ± 494.28	7944.65 ± 49.45	7703.67 ± 389.05	8009.07 ± 418.56	7530.20 ± 310.60					
Mw (kDa)	39103.10 ± 4126.69**	30784.25 ± 2922.95	35094.50 ± 2715.76	40768.00 ± 112.1-0***	37292.60 ± 1794.76*	41044.17 ± 1375.40***	33338.85 ± 2830.05					
M (Avg)	5281.20 ± 552.53	4824.60 ± 13.50	5025.97 ± 208.03	5208.05 ± 66.75	5173.97 ± 533.58	5452.00 ± 363.85	5170.13 ± 418.76					
Mw/Mn	5.01 ± 0.22***	4.36 ± 0.24	4.68 ± 0.16*	5.13 ± 0.02***	4.86 ± 0.47**	5.14 ± 0.42***	4.51 ± 0.24					
Rw (nm)	64.87 ± 3.42	62.17 ± 0.65	62.87 ± 4.19	64.50 ± 3.65	64.30 ± 3.92	63.00 ± 3.32	66.97 ± 2.54					
Rz (nm)	102.57 ± 7.62	96.77 ± 1.72	97.13 ± 8.26	100.17 ± 7.72	98.87 ± 9.61	97.40 ± 6.33	101.53 ± 1.85					
R (Avg)	66.00 ± 2.33	62.50 ± 2.77	63.87 ± 2.27	64.30 ± 2.35	64.97 ± 2.63	64.13 ± 1.46	66.05 ± 0.95					
Rh (Q)	42.37 ± 0.93	41.57 ± 0.06	42.30 ± 0.26	42.13 ± 0.47	42.47 ± 0.61	41.33 ± 1.17	42.63 ± 0.61					
Polymers mass fraction (%)	14.37 ± 1.71*	10.05 ± 0.55***	13.01 ± 2.26***	14.83 ± 0.84*	16.37 ± 0.85	13.48 ± 0.63***	14.42 ± 0.68*					
SEC												
UPP/EPP	1.14 ± 0.13	1.09 ± 0.00	1.05 ± 0.05	0.89 ± 0.13**	0.87 ± 0.07**	1.1 ± 0.00	1.13 ± 0.14					
UPP/TPP (%)	53.02 ± 2.93	52.13 ± 2.93	51.25 ± 1.15	46.99 ± 3.53*	46.58 ± 1.89*	52.45 ± 0.08	52.88 ± 3.11					
Glii/Glis	0.81 ± 0.16	0.82 ± 0.01	0.83 ± 0.08	0.56 ± 0.15***	0.62 ± 0.06**	0.81 ± 0.02	0.76 ± 0.06					

14% humidity; RT - room temperature; HMW-GS - high molecular weight glutenin subunits; LMW - low molecular weight glutenin subunits; Free SH - free sulphydryl groups; R_{max} - maximum resistance to extension; Ext - total extensibility; ν - slope; Mn - number average molar mass; Mw - weight average molar mass; M(Avg) - average molar mass; Mw/Mn - polydispersity index; Rw - gyration radius in mass; Rz - gyration radius; R (Avg) - average radius; Rh - hydrodynamic radius; UPP - unextractable polymeric protein; EPP - extractable polymeric protein; Glii - unextractable gliadin protein; Glis - extractable gliadin protein; TPP - Total polymeric protein.

Table 2Regression coefficients for the response surfaces of microwave treatment of wheat gluten. Significant values are given in bold as $* p \leq 0.05$.

Source	Temperature	Moisture content	Gliadins	Glutenins	Free SH	R5 reactivity	R _{max}	Ext
Intercept	58.05*	7.12*	60.50*	11.92*	5.28*	271407.7*	100.60*	59.14*
X ₁ (b ₁)	10.11*	-0.58*	2.91*	-0.074	-0.099	1637.0	18.06*	1.81*
X ₁ ² (b ₁ ²)	1.78*	-0.055	-2.83*	-0.12	0.030	2159.0	-0.67	-4.34*
X ₂ (b ₂)	4.26*	-0.10	3.67*	-0.56	0.076	-1047.1	-5.11*	-2.17*
X ₂ ² (b ₂ ²)	0.33	-0.027	-2.71*	0.27	-0.050	-544.1	4.33	-6.63*
X ₁ X ₂ (b ₁₂)	2.10*	-0.043	1.41	0.42	-0.035	11872.0	11.20*	-6.87*
R ²	0.925	0.732	0.721	0.434	0.356	0.0897	0.706	0.9325
R ² (adjusted)	0.871	0.541	0.521	0.0304	0.000	0.000	0.496	0.884

**Fig. 1.** The response surfaces of the impact of power level and treatment time on the maximum temperature achieved (A), moisture content (B) and the content of extractable gliadins (C).

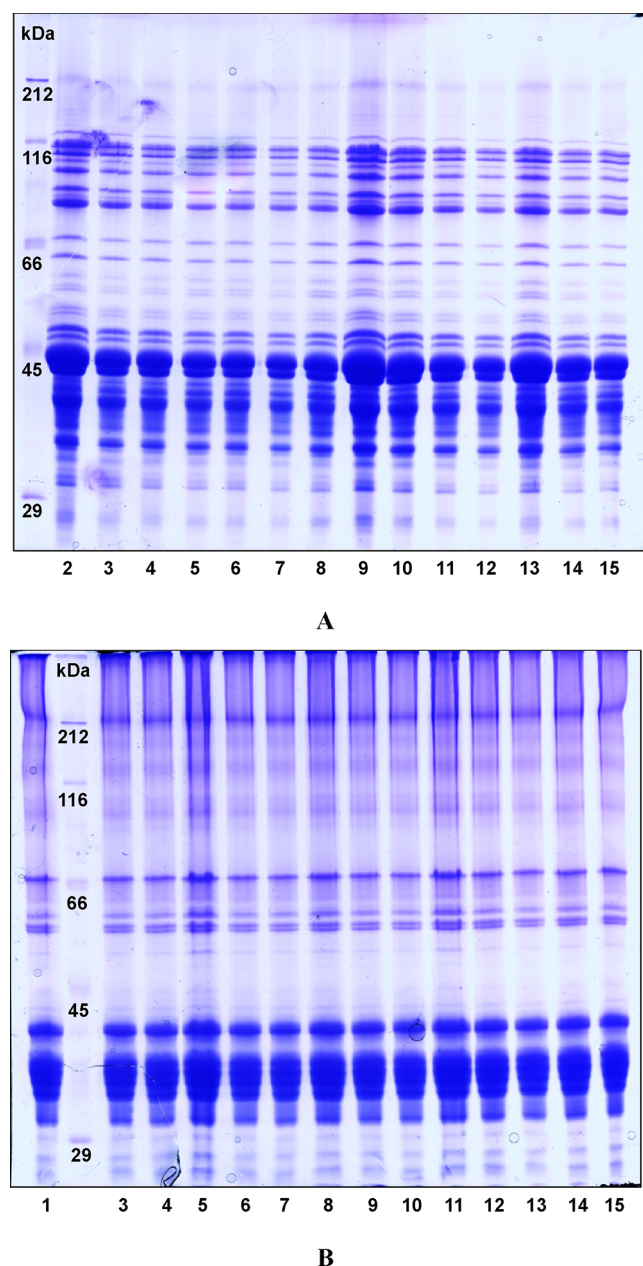


Fig. 2. Reduced and alkylated glutenin subunit patterns (A) and gliadin patterns (B) of gluten, control and microwave treated, analyzed by SDS-PAGE using 12% and 10% gels, respectively, for high molecular weight-glutenin subunits (HMW-GS), low molecular weight-glutenin subunits (LMW-GS), ω - and $\alpha/\beta/\gamma$ -type gliadins. Lane 2 (A) and Lane 1 (B), control gluten (Power 0 W, Time 0 s). Lane 3, microwave treated gluten 1 (317.1 W, 25.85 s). Lane 4, gluten 2 (882.8 W, 25.85 s). Lane 5, gluten 3 (317.1 W, 54.14 s). Lane 6, gluten 4 (882.8 W, 54.14 s). Lane 7, gluten 5 (200 W, 40 s). Lane 8, gluten 6 (1000 W, 40 s). Lane 9, gluten 7 (600 W, 20 s). Lane 10, gluten 8 (600 W, 60 s). Lane 11, gluten 9 (600 W, 40 s). Lane 12, gluten 10 (600 W, 40 s). Lane 13, gluten 11 (600 W, 40 s). Lane 14, gluten 12 (600 W, 40 s). Lane 15, gluten 13 (600 W, 40 s). Sizes (in kilodaltons) of protein molecular weight markers are shown (Lane 1-A and Lane 2-B).

increasing the $R_{\max}$ in others.

For the power levels used, the linear effect was found significant. The microwave treatment time did also influence the $R_{\max}$, but in this case, only the quadratic effect was found significant. It was also observed a significant interaction between these two parameters (Fig. 3A). Overall, the maximum value of $R_{\max}$ was found when the gluten was treated at the maximum power level and treatment time, being

observed a significant correlation ($r = 0.561$, $p < 0.046$) with the maximum temperature achieved during the microwave treatment. These results are partially comparable with the results reported in (Warchalewski, Gralik, Grundas, Pruska-Kędzior, & Kędzior, 2011) who found that the microwave treatment of the gluten increases its elastic modulus.

For the Ext, the microwave treated glutes presented a significantly different value when compared to the control gluten (Table 1). On average, excluding the gluten 2 and the central points, there was a decrease in Ext. Gluten 2 was the only case where it was observed an increase in Ext (Table 1). For the power levels and treatment times used, both the linear and quadratic terms were significant, being also observed a significant interaction between both factors (Fig. 3b).

Disulphide bonds play a fundamental role in determining the polymeric structure and functional properties of gluten (Shewry, Halford, Belton, & Tatham, 2002). Regression analysis of the results obtained for the free sulfhydryl content of the microwave treated glutes did not allow to obtain a good model in the range of power levels (200–1000 Watt) and treatment times studied (20–60 s; Table 1). The free sulfhydryl content of the control gluten was $5.19 \pm 0.12 \mu\text{mol/g}$ of protein. After microwave treatment, the free sulfhydryl content ranged from 4.87 ± 0.07 to $5.59 \pm 0.07 \mu\text{mol/g}$ of protein (Table 1). The glutes 3 and 5 presented a significantly higher content of free sulfhydryl groups when compared to control gluten. The gluten 2 presented a lower content of free sulfhydryl groups. Interestingly, the glutes 3 and 5 were those glutes with a lower $R_{\max}$ whereas gluten 2 was the gluten with the higher value of $R_{\max}$. In fact, a significant negative correlation between the content of free sulfhydryl groups of glutes and the $R_{\max}$ ($r = -0.727$, $p < 0.005$) was observed. Nevertheless, the variation in the content of free sulfhydryl groups only explained 53% of the total variation observed for the $R_{\max}$ when gluten was treated by microwave. The generation of free radicals due to the absorption of the microwave energy by the polar groups of proteins can disrupt various interactions among protein molecules, including Van der Waals forces, electrostatic and hydrophobic interactions, H-bonding and disulphide bonds, modifying in this way the secondary and tertiary structure of proteins and their functional properties (Han et al., 2018).

In order to understand the effect of microwave treatment in the gluten secondary structure, the samples were analysed by FTIR. The deconvolution of the amide I band of control and microwave treated glutes, allowed to analyse the main secondary structure elements, namely the relative intensities at ~ 1671 (β -turns), 1657 (α -helix and random), 1628 (β -sheet) and 1614 cm^{-1} (intermolecular β -sheet) (Wellner et al., 2005). The relative intensities of the gluten main secondary structure elements are summarized in Table 1. The intensities of the bands were divided by the intensity of the band at 1644 cm^{-1} in order to avoid the normalization factor. The results clearly show that the secondary structure of gluten was modified by the microwave radiation. The α -helix (band at 1657 cm^{-1}) structures were significantly affected by microwave treatment. In fact, these structures are associated with the N- and C-terminal regions of the high molecular weight glutenin subunits (HMW-GS) that contain cysteine residues (Li, Dobraszczyk, Dias, & Gil, 2006). Considering that the heating generated by the microwave treatment promotes sulfhydryl-disulphide interchange reactions, the decrease in the relative abundance of these secondary structures suggests a different arrangement of the disulphide bonds in the microwave treated glutes. Furthermore, the relative intensity of the β -turn structures (1671 cm^{-1}) was also affected by the microwave treatment, but the effect, in this case, seems to be more random between the samples treated by the microwave with the different power levels and treatment times. It was proposed that the β -turn structures of the HMW-GS are organized in a regular β -spiral structure (Shewry et al., 2002). Thus, the decrease or increase in the relative intensities of the β -turn structures observed for the samples treated with the microwave in relation to the control gluten suggests the occurrence of changes in the β -spiral structure and/or in its proportion (Wellner

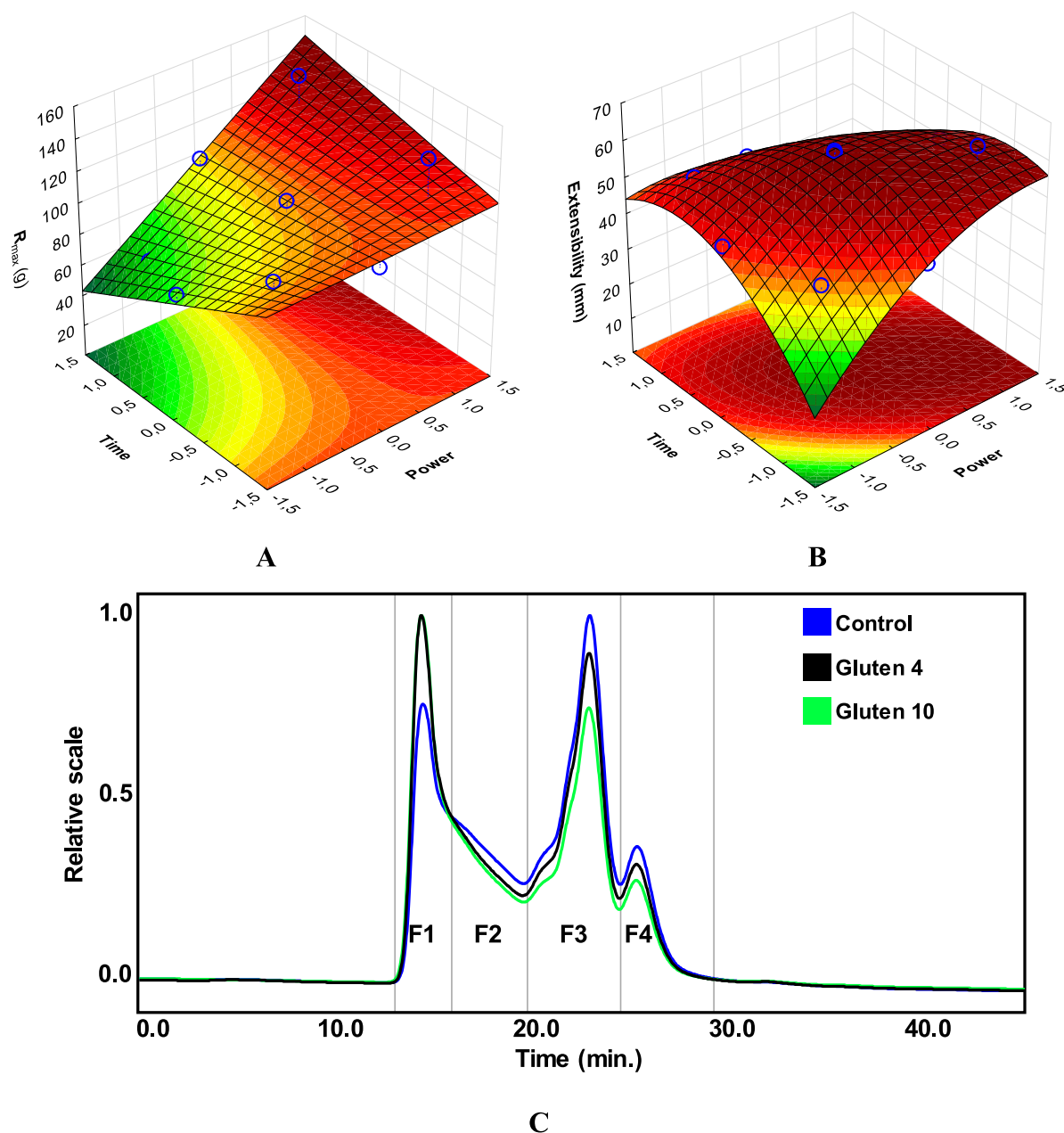


Fig. 3. The response surfaces of the impact of power level and treatment time on the maximum resistance to extension (R_{max} ; A) and extensibility (Ext; B) of glutes. SE-HPLC protein profile of extractable proteins from control gluten, gluten 4 and gluten 10 at 214 nm (C). The elution profile was divided into four main peaks corresponding to polymeric glutenin proteins (F1s + F2s), gliadins (F3) and functional proteins (F4).

et al., 2005). Considering the importance of this structure for the elastic behavior of gluten (Humphris et al., 2000; Tatham, Mifflin, & Shewry, 1985), we can state that in addition to the changes promoted by the microwave treatment at the level of tertiary structure imposed by the disulphide bonds, the changes in the secondary structure of the gluten proteins may also have an important role in defining the rheological and functional properties of the microwave treated gluten. The modification of the gluten proteins induced by the microwave radiation is also confirmed by the change in abundance and molecular features of the polymers determined by asymmetrical flow field-flow fractionation (Table 1). The microwave treatment of gluten induced changes in the molecular weight distribution of the polymeric aggregates, which was mainly substantiated by the weight-average molar mass (Mw) and the dispersity (Mw/Mn) (Ribeiro et al., 2019). The Mw and dispersity of the polymeric aggregates significantly increased for the majority of the microwave treated glutes, namely glutes 6, 7, 10, 11 and 12, and 3,

6, 7, 9, 10, 11 and 12, respectively, also increasingly on average for both parameters. The observed changes in the molecular distribution of the glutenin polymers were accompanied by a significant decrease in the aggregate mass fraction percentage showing disruption of weak interactions (such as hydrophobic, Van der Waals and hydrogen interactions) between the aggregated polymers. In addition, considering the absence of significant effects on the content of free sulfhydryl groups, the changes in Mw were not due to glutenin polymerization and/or depolymerization but due to protein disaggregation.

To better understand the effect of microwave treatment on the functionality of gluten and on the macromolecular features of proteins, the SDS protein solubility was studied using SEC. Parameters such as UPP/EPP, UPP/TPP and Glii/Glis which play a key role in the formation of polymeric aggregates and the arrangement of the gluten network were determined (Table 1 and Fig. 3C).

The microwave treatment significantly modified the 2% SDS

solubility of gluten proteins. UPP/EPP, UPP/TPP, and Glii/Glis parameters decreased for all the microwave treated samples in relation to the control gluten. The UPP/EPP ratio decreased from 1.20 to 0.65 for the control gluten and microwave treated gluten 6 (where the higher temperature was achieved), respectively. The UPP/TPP (%) decreased from 54.26% to 39.39% for the control gluten and microwave treated gluten 6, respectively. These changes were due to a disaggregation phenomenon of the polymeric aggregates. The most affected fraction was the unextractable polymeric protein, namely fraction 2 of the unextractable glutenin (Table 1). At the same time, the microwave treatment significantly modified the unextractable gliadins to extractable gliadins ratio (Glii/Glis). As expected, gliadin solubility increased significantly with treatment. The Glii/Glis ratio decreased from 0.94 to 0.39 for the control gluten and microwave treated gluten 6, respectively. These decreases mean that the interactions between gliadin and polymeric aggregates were disrupted leading to the changes observed in the molecular weight distribution of the polymeric aggregates.

3.3. Effect of microwave treatment on gluten potential celiac-immunogenicity

The effects of power level and treatment time of microwave treatment on the amount of potential celiac-toxic epitopes released after simulated gastric and intestinal digestion were evaluated and are reported in Table 1 and Fig. 4. No significant linear, quadratic and interaction effects of the factors analysed were observed. Nevertheless, it should be noted that none of the microwave treated samples showed on average an inferior content of toxic epitopes than the control gluten. The values obtained ranged from 227.5 ± 7.3 g/kg of the control gluten to 298.8 ± 3.5 g/kg of the microwave treated gluten 12 (Table 1 and Fig. 4). These results clearly show that despite the significant differences observed in relation to the gluten secondary structure and polymeric macromolecular features, the microwave treatment cannot decrease the amount of potential celiac-toxic epitopes released after simulated gastric and intestinal digestion. These results are partially in accordance with those of Leszczynska et al. (2003) and Gianfrani et al. (2017) who observed that the microwave treatment didn't reduce the celiac-immunogenicity of gliadins from a commercial preparation and from *T. durum* and *T. aestivum* soaked kernels, respectively. Nevertheless, Gianfrani et al. (2017) observed a decrease in the R5 reactivity when the undigested alcohol-soluble proteins obtained from the microwave treated kernels were analysed using the R5-sandwich enzyme-linked immunosorbent assay (ELISA), which contrasts with their results from the G12 antibody-based ELISA, mass spectrometry-based proteomics and *in vitro* assay with T-cells of celiac subjects, where

no differences in toxicity between the microwave treated and control kernels were found. In our work we used the peptic-tryptic digests of the microwave treated glutes and the R5-competitive ELISA format for measuring the potential celiac-immunotoxicity of the microwave treated gluten (Kahlenberg et al., 2006; Osman et al., 2001) and we observed that there was an average increase in the gluten toxicity (+20%) after microwave treatment. On the other hand, Lamacchia et al. (2016) reported that the microwave treatment of wheat kernels, a mixture of soft and durum wheats, at 1000 Watt during 2 min reduced the cross-reactivity with the R5 antibody by 99% which is in accordance with the above-mentioned results reported by Gianfrani et al. (2017) when using the R5 antibody without previous proteolysis. Both results seem to clearly point out that the measurement of the potential immunogenicity of gluten using antibody assays should be performed with previously hydrolysed material. The increase in celiac-immunotoxicity observed for the microwave treated gluten (maximum 31%) can be explained by the disaggregation of the gluten proteins as discussed previously (Table 1) where it was observed a significant increase in the SDS-extractable gluten proteins, including gliadins, in the microwave treated glutes, nevertheless, other factors seem to be also affecting the release of the R5 reactive epitopes by the proteases as there was observed a significant Spearman correlation between the amount of R5 reactive epitopes and the content of free sulphhydryl groups ($\rho = 0.560$, $p < 0.05$) and extractable HMW-GS ($\rho = 0.534$, $p < 0.05$). These changes can render a gluten protein structure more accessible to proteases.

4. Conclusions

The results obtained in this work clearly shows that the microwave treatment of gluten proteins cannot decrease its celiac-related toxicity, on the contrary, it can render the gluten with a potential increased immunotoxicity for people who suffer from celiac disease. This can be due to an increase in gluten digestibility due to the disaggregation phenomenon observed with the microwave treatment. Nevertheless, from a functional point of view, microwave treatment can be an efficient treatment to increase the R_{max} in a proper combination of treatment time and power level and can be used advantageously for the production of vital gluten. This increase in R_{max} is due to a decrease in the content of free sulphhydryl groups and rearrangement of disulphide bonds as well as an increase in the abundance of polymers. Besides the sulphhydryl-disulphide interchange reaction, the changes in the secondary structure of gluten proteins are also important to explain this effect.

Declaration of Competing Interest

None.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodchem.2019.124986>.

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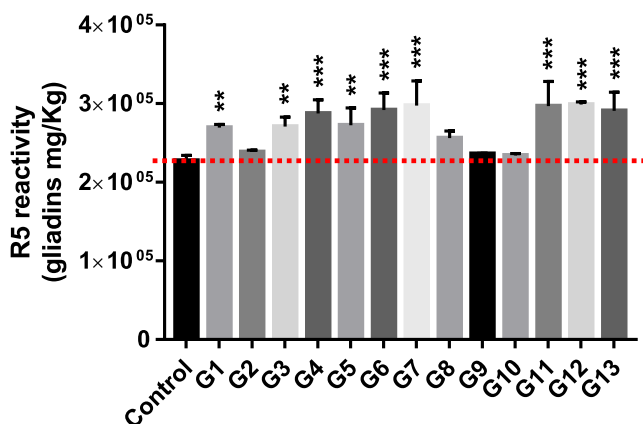


Fig. 4. R5 reactivity of the different microwave treated glutes and control measured after peptic and tryptic digestion. Differences in relation to the control are given as * $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$. Control – control gluten; G1 to G13 – microwave treated gluten. For more details on G1 to G13, please consult Table 1.

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Supplementary material

How microwave treatment of gluten affects its toxicity for celiac patients? A study on the effect of microwaves on the structure, conformation, functionality and immunogenicity of gluten

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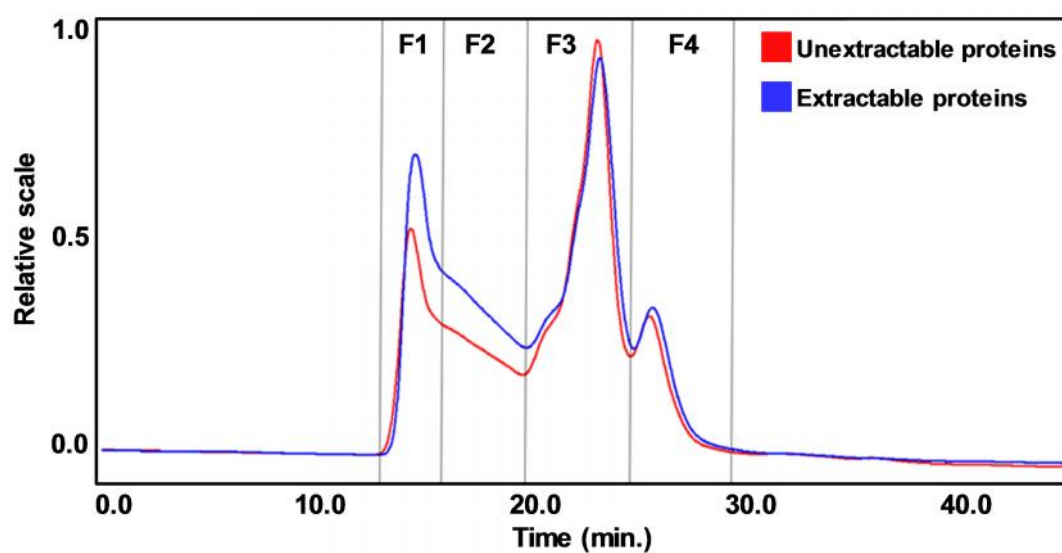
Both authors contributed equally to this project

Supplementary Table 1. Experimental levels of the two independents variables.

Independent variables	Mathematical symbols	Coded and uncoded values				
		-1.414	-1	0	1	1.414
Power (Watt)	X_1	200	317.1	600	882.8	1000
Time (s)	X_2	20	25.85	40	54.14	60

Supplementary Table 2. Experimental design of 2 factors 5 levels Central composite design (CCD) of microwave treatment.

Experiments	Power (Watt) (X_1)		Time (s) (X_2)	
	Coded value	Uncoded value	Coded value	Uncoded value
1	-1	317.1	-1	25.85
2	1	882.8	-1	25.85
3	-1	317.1	1	54.14
4	1	882.8	1	54.14
5	-1.414	200	0	40
6	1.414	1000	0	40
7	0	600	-1.414	20
8	0	600	1.414	60
9	0	600	0	40
10	0	600	0	40
11	0	600	0	40
12	0	600	0	40
13	0	600	0	40



Supplementary Figure 1. SE-HPLC protein profile of control gluten at 214 nm. Blue line and red line denote SDS-soluble and insoluble protein extracts of control gluten, respectively. The elution profile was divided into four main peaks corresponding to polymeric glutenin proteins (F1s+F2s), gliadins (F3) and functional proteins (F4).