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Title

Understanding the influence of buckwheat bran on wheat dough baking performance: Mechanistic insights from molecular and material science approaches

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Highlights

- Molecular and material sciences provide quantitative description of mechanisms behind bran functionality
- Bran addition induces changes in gluten structural arrangements due to reduced gluten solvation
- Bran induced changes in water partitioning in dough also control starch gelatinization
- Dough baking performance, i.e. specific volume, is controlled by gluten structural arrangements
- Crumb density and the volume fractions of water and bran modulate crumb texture

Keywords

Buckwheat bran, thermo-mechanical behaviour, water distribution, cellular solids, gluten structure, baking

Abbreviations

WF, wheat flour dough/bread; CB5 dough/bread with 5% coarse buckwheat bran and 95% wheat flour; CB10 dough/bread with 10% coarse buckwheat bran and 90% wheat flour; CB20 dough/bread with 20% coarse buckwheat bran and 80% wheat flour**;** FB5 dough/bread with 5% fine buckwheat bran and 95% wheat flour; FB10 dough/bread with 10% fine buckwheat bran and 90% wheat flour; FB20 dough/bread with 20% fine buckwheat bran and 80% wheat flour.

Abstract

A molecular and material science approach is used to describe the influence of coarse and fine buckwheat bran on wheat dough properties and bread textural quality. Focus is given on (i) gluten solvation and structural arrangements in presence of bran as studied by front-face fluorescence; (ii) thermo-mechanical behavior of dough during heating studied by dynamic mechanical thermal analysis and (iii) texture of bread crumb analyzed in terms of a cellular solid. The thermo-mechanical behavior of dough was found to be largely related to starch phase transitions during heating. The use of thermodynamic approaches to biopolymer melting revealed that key transitions such as the onset of starch gelatinization were function of the interplay of water and bran volume fractions in the dough. Front-face fluorescence studies in wheat dough revealed that gluten solvation and structural arrangements were delayed by increasing bran addition level and reduction in particle size, as indicated by the drastic decrease in the protein surface hydrophobicity index. Variations in gluten structure could be strongly related to dough baking performance, i.e. specific volume. With regards to texture, the approach revealed that crumb texture was controlled by variations in density, moisture and bran volume fractions. Overall, this study elucidates a number of physical mechanisms describing the influence of buckwheat bran addition to dough and bread quality. These mechanisms strongly pointed at the influence of bran on water partitioning among the main polymeric components. In the future, these mechanisms should be investigated with bran material of varying source, composition and structure.

1. Introduction

In the last decades, the demand for healthy foods has grown due to increased consumers awareness of the role of nutrition in preventing or lowering the risk of developing chronic diseases such as cardiovascular disease, cancer or type 2 diabetes (Montagnese et al., 2015; Who & Consultation, 2003). Cereal and pseudo-cereal products, being a staple food category, may represent a valid resource to provide adequate amount of nutrients such as non-digestible cell wall polymers, i.e. dietary fiber, and related compounds with relevant bio-activity (Vitaglione, Napolitano, & Fogliano, 2008).

Common buckwheat (*Fagopyrum esculentum*) is a nutritional-relevant pseudo-cereal, being an important source of dietary fiber and antioxidant compounds (Steadman, Burgoon, Lewis, Edwardson, & Obendorf, 2001 a). In particular, buckwheat is rich in polyphenols, including the flavonoid rutin, which has been studied as a potential health protective compound thanks to its anti-inflammatory and anticarcinogenic activity (Zhang et al., 2012). Moreover, buckwheat proteins have high biological value and balanced amino acid composition containing a relatively high amount of lysine – the limiting amino acid in wheat – (Dziadek et al., 2016).

Buckwheat flour is traditionally used in a number of products including pancakes (Mazza and Dave Oomah, 2005), crêpes (Biacs, Aubrecht, Léder, & Lajos, 2002), Italian pasta "Pizzoccheri" and noodles, *e.g.* Soba in Japan (Bonafaccia, Marocchini, & Kreft, 2003; Marti, Fongaro, Rossi, Lucisano, & Ambrogina Pagani, 2011; Pagani, Lucisano, & Mariotti, 2007). Recently, buckwheat flour has gained popularity as a functional ingredients in bread in order to obtain an economically advantageous enrichment in naturally derived antioxidants (Dziki, Rózyło, Gawlik-Dziki, & Świeca, 2014; Vogrinčič, Timoracka, Melichacova, Vollmannova, & Kreft, 2010).

The outermost layers of buckwheat groats contain most of the nutritional compounds (Steadman et al., 2001 b) but they are usually discarded during the production of refined flour and collected into feeding material. The enrichment of wheat-based products with buckwheat bran provides an opportunity to improve nutritional profile and valorize the side stream material.

Many studies deal with the technological impact of wheat bran on bread quality, indicating detrimental effects resulting in decreased loaf volume, increased crumb hardness and changes in sensory properties (Ktenioudaki & Gallagher, 2012). The observed negative effects have been largely associated with changes in gluten development and quality (Heiniö et al., 2016; Schmiele, Jaekel, Patricio, Steel, & Chang, 2012; Sivam, Sun‐Waterhouse, Quek, & Perera, 2010). The mechanisms by which bran negatively impacts dough quality have been ascribed by authors to gluten dilution (Gan, Galliard, Ellis, Angold, & Vaughan, 1992) and physical hindrance (Lai, Hoseney, & Davis, 1989), decreased gluten development due to bran competition for water (Hemdane, Jacobs, et al., 2016), and by chemical interactions between wheat bran components and gluten proteins which affects network formation (Noort, van Haaster, Hemery, Schols, & Hamer, 2010). Furthermore, Campbell et al. (2008) suggested that bran acted during baking rather than during proofing by releasing extra water available for starch gelatinization, and thereby lowering the final bread volume.

Modulating bran particle size has been indicated as a tool to optimize the baking quality of bran enriched breads. Reduction in bran particle size has been often associated with more detrimental effects on dough and bread quality compared to coarse bran. The detrimental effects have been related to an increased particle surface leading to more chemical interactions with gluten and liberation of reactive compounds (Noort et al., 2010). On the contrary, some researchers have suggested an increase bread volume with reducing particle size (Lai et al., 1989) or even no significant effects (Coda, Rizzello, Curiel, Poutanen, & Katina, 2014). Bran type, addition level and breadmaking protocols are likely to explain the observed differences (Hemdane, Jacobs, et al., 2016).

While many studies have addressed gluten quality focusing on yield, the impact of bran on gluten structure at molecular level has not yet been fully addressed. Furthermore, only limited studies have looked at mechanisms by which bran affects the thermo-mechanical behavior of dough and crumb texture. Few authors studied the effect of buckwheat bran on wheat bread quality

(Fujarczuk & Zmijewski, 2009; Atalay, Bilgicli, Elgün, & Demir, 2013), but information on the its technological impact on wheat dough properties is still scarce. For such reasons, the present study aimed at evaluating the mechanisms by which addition of buckwheat bran impacts gluten structure, thermo-mechanical transitions during baking and consequently bread texture. For this purpose, coarse and fine buckwheat bran of similar composition were added at different levels on wheat dough. Front-face spectroscopy in flour-water mixtures was performed to elucidate the effect of bran addition on gluten structural arrangements and gluten solvation. Dynamic mechanical thermal analysis was performed to investigate the influence of bran on dough rheology and phase transitions. The insights on dough properties were complemented with the evaluation of baking performance and textural quality of bread during four days storage. The mechanisms by which buckwheat bran impact dough thermo-mechanical behavior and crumb texture have been analyzed by means of a material science approach (Renzetti & Jurgens, 2016).

2. Materials and methods

2.1 Materials

Common buckwheat (Fagopyrum esculentum) bran was provided by Filippini s.p.a. (Teglio, Italy) as coarse bran (CB). Part of the supplied bran was processed in a micronizer system (KMX-300i; Separ Microsystem, Brescia, Italy) to reduce the particle size and to obtain a fine bran (FB). Proximate composition of coarse and fine buckwheat bran fractions with regards to ash, protein, total starch, soluble and insoluble dietary fiber are provided in Table S1 (supplementary material). The average particle size (in diameter) of CB and FB was respectively 359 and 113 μm as measured by sieving method. A commercial wheat flour (WF) for bread making application (protein: 10.6 g/100 g) was provided by Meneba (Rotterdam, The Netherlands).

2.2 Bran sorption properties

The moisture sorption behavior of coarse and fine bran was evaluated in duplicate according to Erickson, Renzetti, Jurgens, Campanella, & Hamaker (2014) by using an automatic multi-sample moisture sorption analyzer SPSx-11l (Projekt Messtechnik, Ulm, Germany).

Water Binding Capacity (WBC) of coarse and fine bran was assessed by soaking 1.5 g of bran in 45 mL of Milli Q water and shacked for 16 h at room temperature. After soaking, samples were centrifuged for 60 min at 10000. after which the supernatant was discarded from the pellet. The samples were then left to drain for 15 min by placing the tubes at an angle of 45°. The residue was weighed and the WBC was calculated by subtracting the initial sample mass. At least 3 replicates were carried out for each sample.

2.3 Definition of dough mixing conditions

Doughs were prepared with coarse or fine bran by adding 5 g, 10 g, and 20 g of bran to 95, 90 and 80 g of flour, respectively. Coarse bran-enriched doughs were labeled as CB5, CB10, and CB20, whereas micronized bran-enriched doughs were labeled as FB5, FB10 and FB20 with numbers indicating the level of addition. A reference dough with no addition of bran was prepared as control (WF). The required water absorption for comparable dough consistencies in the bread-baking test was determined in a Farinograph-E (Brabender, Duisburg, Germany) equipped with a 50 g mixing bowl. The ICC standard method 115/1 (ICC-Standards, 2006) was used with few modifications. Briefly, 50 g of wheat flour or buckwheat bran-enriched mixture were added of 1 g of sodium chloride (Merck, The Netherlands) and pre-mixed for 2 min. Water addition levels were defined by running an appropriate number of replicates until the maximum dough development was centered on the 420 FU (Farinograph Units), according to TNO established method.

2.4 Protein structural data of dough

Protein surface hydrophobicity was assessed through titration of doughs prepared with increasing concentrations of 1,8-aniline-naphtalen sulfonate (ANS) as reported by Bonomi et al. (2004). Water was added to flour or to flour-bran mixtures at the absorption levels indicated by farinograph tests. Front-face (solid state) spectrofluorimetric measurements were carried out in an LS-50

spectrofluorimeter (Perkin-Elmer Waltham, MS) by recording emission fluorescence spectra (from 400 to 600 nm, with excitation at 390 nm, emission and excitation slits set at 5 nm) on small amounts of individual dough samples containing 0-0.5 mmol L-1 ANS. Individual samples were prepared by carefully hand-mixing of flour (3 g) and water (containing the required amount of ANS) with a glass rod for 3 minutes. Preparations trials indicated no changes in the fluorescence intensity or in the spectra shape were observed for manual mixing times longer than 3 minutes, as previously reported (Huscka et al., 2012). The resulting mass was cut and placed behind a quartz window in the measuring cell. The cell was tightly closed to cover the entire window by spreading the sample.

Standard binding algorithms were used to calculate Fmax (i.e., the fluorescence at saturating probe concentration, related to the number of surface hydrophobic sites available for binding of the probe), and Kd (i.e., the apparent dissociation constant of the assumedly bi-molecular probe/protein complex) from the ANS titration data. Fluorescence intensity at saturating ANS concentration was corrected for the protein content in the dough.

These two parameters were combined in a protein surface hydrophobicity index (PSH), calculated as the ratio (Fmax/protein content)/Kd (Bonomi et al., 2004). Samples were prepared in duplicate for each bran type, bran addition level and ANS concentration.

A similar solid-state spectrofluorimetric approach was used to assess the extent of protein solvation in dough samples containing 0.3 mM ANS and prepared by mixing at water contents ranging from 40 to 55%, as also described in Bonomi et al. (2004). All samples for spectrofluorimetric measurements were prepared in duplicate, and multiple emission spectra (n = 3) were averaged for each individual sample.

2.5 Thermo-mechanical behavior and phase transitions in dough

2.5.1 Dynamic Mechanical Thermal Analysis (DMTA) of dough

Dough viscoelastic properties were measured by using a DHR2 hybrid rheometer (TA Instruments, New Castle, USA) equipped with 25 mm steel parallel Peltier plate. Approximately 1 g

of dough (prepared without yeasts) was placed between plates (loading gap: 20 mm) and compressed until 1.025 mm. Dough excess was removed and silicon oil was applied to prevent sample drying and dough was compressed until 1 mm. Before the measurement, the dough was rested for 5 min at 25 °C to allow relaxation. Samples were oscillated at a frequency of 1 Hz and heated from 40 to 120 °C with a ramp of 5 °C/min. Before analysis, oscillation amplitude test was performed from 1.0e-4 to 10 to select the linear viscoelastic range. Thus the strain amplitude was kept at 0.5e-3 for all samples. Key parameters related to physical transitions in the dough were derived from the analysis of the G´ and tan(δ) curves in the DMTA curves by using the analysis functions in TA Trios v3.3 (TA Instruments, New Castle, USA): onset temperature of starch gelatinization, T_{onset}, from evolution of G´ during heating (calculated as the intersection of the tangents of the baseline before the sudden increase in G´ and the tangent of the steep G´ profile after T_{onset}); tan(δ) value at onset G´; G´ at peak ($G´_{max}$) and the temperature corresponding to $G´_{max}$ (T_{max}). The analysis was carried out at least in triplicate on independent doughs.

2.5.2 Differential Scanning Calorimetry (DSC)

Starch gelatinization in doughs was measured using a DSC Q200 (TA Instruments, New Castle, USA). Samples (10-15 mg) were placed in sealed aluminum pans, equilibrated at 2 °C for 5 min, and scanned to 160 °C at a rate of 7.5 °C/min. Starch gelatinization temperatures (onset, maximum peak) were determined by using the analysis functions in Universal Analysis software (TA Instruments, New Castle, USA). The analysis was carried out at least in triplicate on independent doughs.

2.5.3 Theoretical background for a quantitative description of the influence of dough composition on starch gelatinization

According to the Flory-Huggins equation for biopolymer melting, the starch gelatinization temperature in a water solution is function of the volume fraction of water (Φ_{water}) present in the food matrix (Renzetti & Jurgens, 2016), following the equation:

$$\frac{1}{T_m} - \frac{1}{T_m^\circ} = \frac{R}{\Delta H_U}\frac{v_U}{v_W}[\Phi_{water} - \chi\Phi_{water}^2] \qquad (1)$$

Where T_m is the melting temperature of starch in the system under consideration, T_m° the melting temperature of the dry crystalline starch, ΔH_U is the melting enthalpy per mole of the repeat unit of the biopolymer, i.e. starch, v_U is the molar volume of the starch repeat unit, v_{water} is the molar volume of the diluent, i.e. water, Φ_{water} is the volume fraction of water, χ is the Flory-Huggins solvent-biopolymer interaction parameter and R is the universal gas constant. The theory can also be applied to a system composed of water and flour since the ratio between gluten and starch is constant and hence water will partition between the two components in a similar manner, irrespective of its volume fraction. However, the addition of bran changes the partitioning of water in the system as it will compete with starch and gluten to absorb the available water. According to Flory-Huggins theory, the partitioning of water can be described as the chemical potential of water among the different polymer phases following (Van der Sman & Meinders, 2011):

$$\frac{\mu_w}{RT} = ln(1-\Phi) + \left(1 - \frac{1}{N}\right)\Phi + \chi\Phi^2 \qquad (2)$$

Where μ_w is the chemical potential of water, Φ the volume fraction of the biopolymer, N is the ratio of the molar volume of biopolymer and water and χ is the interaction parameter water-biopolymer. From the equation, it follows that the partitioning of water will change with increasing volume fraction of bran Φ_{bran}, thus reducing the amount of water available for starch gelatinization. When such approach holds for the wheat dough system and the χ of bran is unaffected by micronization (i.e. the moisture sorption properties are similar for fine and coarse bran), the onset of starch gelatinization should be mainly a function of both Φ_{water} and Φ_{bran}. However, it should be taken into account that in a complex system like the wheat dough under study, the variation in water volume fraction Φ_{water} are not fully representative of the variation in the water to starch ratio as in the case of the water-starch system described by equation (1). For such reason, Φ_{water} should be rescaled over the volume fraction of starch Φ_{starch} in dough by using the following equation:

$$\Phi_{ws} = \frac{\Phi_{water}}{\Phi_{water} + \Phi_{starch}} \qquad (3)$$

In order to validate the proposed interpretation of data, the volume fraction of ingredients in the dough formulations were computed from the mass fraction using the mass density ρ_i of each ingredient (water: ρ_{water} 1000 kg/m^3; polysaccharide: $\rho_{polysaccharide}$ 1550 kg/m^3; proteins: $\rho_{proteins}$1330 kg/m^3 (Van der Sman, 2008). For bran the mass density was assumed that of polysaccharides.

2.7 Bread making and baking quality evaluations

Small-scale puffy loaves were produced according to Hemery et al. (2010) with slight modifications. Dough was prepared in a 300 g Farinograph mixing bowl at 20 °C and speed of 63 rpm. Instant yeast (1.76%; Fermipan red, AB Mauri), salt (2%; EFP, Akzo Nobel), and calcium propionate (0.1%; Sigma-Aldrich) were added to either wheat flour or buckwheat bran-enriched mixture and pre-mixed in the mixer bowl for 2 min. Then, distilled water was added according to Farinograph water absorption. After mixing until development time, dough was divided in pieces containing 47.9 g of flour, to correct the loaf weight for the different amount of water added, manually rounded, and fermented two time at 30 °C for 15 min. Subsequently loaves were molded (Betrand Euro 2000, Nevers, France) and placed in loaf tins (top: 10.5*4.5 cm; bottom: 9.5*3.5 cm; height: 3.5 cm). Final proof was carried out in a fermentation cabinet (custom made by TNO) at 30 °C and 90% RH for 40 min, corresponding to the time needed to produce 200 mL CO_2 as measured in a SJA-fermentograph (Nässjö, Sweden). Finally, loaves were baked in a custom made swing oven (TNO) at 230 °C for 20 min. For each variation 4 independent doughs were baked, obtaining a total of 24 loaves. Six loaves, deriving from 2 independent baking tests, were used for each analysis time (i.e. day 0, 1, 2 and 4), as described in the next section.

At the day of baking (day 0), loaf volume and weight were determined after 2 hours cooling at room temperature. Loaf volume and loaf weight were determined on 4 loaves for each variation with a rapeseed displacement method and a technical scale, respectively. Specific volume was calculated as loaf volume divided by loaf weight.

2.8 Bread crumb characterization during storage

After cooling, cylindrical crumb samples of 25 mm diameters were cut out from the centre of bread slices of 20 mm thickness from all the freshly baked breads. Part of the samples were used for characterization at day of baking. The rest of the samples were stored for 1, 2 and 4 days at controlled temperature (18 °C) in sealed polyethylene containers until analysis. This operation was performed in order to assess staling as influenced by starch retrogradation and crumb structure while eliminating the contribution of moisture loss and water migration from crumb to crust.

2.8.1 Texture Profile Analysis (TPA)

Texture Profile Analysis (TPA) was carried out using a texture analyzer (TA-XT2i Texture Analyser, Stable Micro Systems, Surrey, UK) equipped with 30 kg load cell and a 75 mm compression plate. Crosshead speed and trigger force were set respectively to 3.30 mm/s and 9.81 mN. Before testing, sample weight was measured by a technical scale (Mettler Toledo, Tiel, The Netherlands). Crumb specimen (diameter: 25 mm), prepared as described above, underwent two cycles of compression until 40% of deformation. The actual height of the sample recorded by the instrument was used to calculate crumb density, considering the specimen as a cylinder with constant diameter. Twelve crumb samples were analyzed for each storage time.

In cellular solids, the hardness of the material is related to its density, according to the Ashby-Gibson theory (Ashby, 1983). In order to correct the instrumental hardness for variations in density, an adapted Ashby-Gibson theory was applied following:

$$\mathrm{Hardness_{crumb}} = \mathrm{CE_{film}}(\rho)^{\mathrm{n}} \qquad (1)$$

where $Hardness_{crumb}$ is the instrumental hardness, $E_{film}$is the elastic moduli of the solid crumb matrix, ρ is the crumb density, C is a constant and n is the parameter describing the cellular structure, *i.e.* n = 3 for a foam and n = 2 for a sponge. For bakery products, the crumb structure can be assumed to be that of a sponge with n=2 (Le Bleis, Chaunier, Chiron, Della Valle, & Saulnier, 2015; Poutanen, Sozer, & Della Valle, 2014; Renzetti & Jurgens, 2016). In order to derive the corrected hardness values, $CE_{film}$was calculated from each measurement. The average density from all crumb samples was then used to obtain the corrected hardness values.

For further interpretation of texture data, the volume fraction of water and bran, Φ_{water} and Φ_{bran} respectively, in the different bread crumbs were computed from the mass fraction (based on ingredients specs, dough recipes and crumb moisture contents) using the mass density ρ_i of each component, as earlier described.

2.8.2 Moisture content and starch retrogradation in crumb

Moisture content of crumb was measured according to AACC method (44-15.02, 2001). Analysis were performed in four replicates for each storage time. Starch melting enthalpy in crumb during storage was measured according to the procedure earlier described in section 2.5.2. The analysis was carried out on four samples for each storage time.

2.9 Statistical Analysis

Analysis of variance (one-way ANOVA) was used for analyzing dough rheology and baking tests data. Different dough/bread samples were considered as factors for ANOVA. Significant differences among the respective means were determined using Fischer's Least Significant Difference (LSD) test. Linear regression analysis of protein surface hydrophobicity data, (i.e. K_d, F_{max} and PSH) was performed to determine significant contribution of coarse and fine bran addition to the doughs. Similarly, linear regression analysis of dough rheology and textural data as function of composition were also performed. All statistical analyses were performed by using XLSTAT Version 2016.02 (Addinsoft, Paris, France).

3. Results and discussion

3.1 Dough properties

3.1.1 Water absorption and dough development

Addition of buckwheat coarse bran (average diameter: 359 μm) to wheat dough resulted in a progressive increase in water absorption with increasing bran level (Table 1), coupled with a gradual decrease in time to peak (data not shown). On the contrary, the addition of buckwheat fine bran (average diameter: 113 μm) showed only a slight increase in water absorption which was

similar for all dough samples, independently from the level of bran inclusion. No clear trend was observed concerning the dough development time with fine bran (data not shown).

The incorporation of increasing amount of bran generally results in higher water absorption values (Sudha, Vetrimani, & Leelavathi, 2007) due to the increased number of hydroxyl groups of fiber that allow more water interaction through hydrogen bonding (Rosell, Rojas, & De Barber, 2001). In fact, under external stresses as in dough mixing, the water weakly bound is released (Hemdane, Jacobs, et al., 2016) and the water absorption is mainly related to the sorption properties of the material, which are largely dependent on the molecular composition (Van der Sman, 2013).

The chemical characterization of the coarse and fine bran indicated relative small changes in soluble and insoluble fiber composition (Supplementary S1). These changes did not affect the sorption properties of the bran materials, since the isotherms of fine and coarse bran were similar (Supplementary S1).. However, a significant reduction in water binding capacity (WBC) was observed after micronization (Supplementary S1), which is in agreement with recent observations on the decrease in WBC of bran with decreasing particle size (Jacobs et al., 2015). Based on these results, it can be suggested that the differences in water absorption of the dough between coarse and fine bran may be mainly related to the effect of particle size rather than bran composition. Variations in particle size most likely results in differences in the level of bran dispersion and in the kinetics of hydration of the biopolymeric components in the dough, thus affecting the farinograph results (Noort et al., 2010).

3.1.2 Protein surface hydrophobicity studies in dough

Gluten solvation as well as the exposure of protein hydrophobic sites were studied for clarifying the influence of coarse and fine bran on gluten development and dough quality. The number of surface-exposed hydrophobic sites, their accessibility to the fluorescent hydrophobic probe ANS, and their affinity towards the probe were assessed by spectrofluorimetric titration. Dough samples of appropriate composition were prepared adding water according to Farinograph water absorption at increasing ANS concentrations. Data analysis through standard ligand binding

algorithms (such as the Scatchard plot shown in Figure S2 of the supplementary material) gave the binding parameters presented in Table 1.

As previously reported by Bonomi et al. (2004), proteins in common wheat dough have a high number of surface hydrophobic sites available for ANS probe binding, as indicated by the F_{max} of the wheat dough reference (Table 1). Enrichment in coarse buckwheat bran resulted in a gradual decrease of the number of protein sites available for the binding of the probe, but had only a modest effect on their average affinity for the probe (as indicated by the apparent dissociation constant, K_d). On the contrary, addition of micronized buckwheat bran had far more dramatic effects on reducing both F_{max} and K_d, especially when high bran levels were considered (> 10%), thus indicating decreased exposure of hydrophobic sites in gluten and reduced affinity for the probe in the presence of bran. Combining F_{max} and K_d in the PSH surface hydrophobicity index provides information on both aspects related to protein structural arrangements. Upon addition of bran, the PSH index significantly dropped from ~ 196 in wheat dough to ~ 44 in the presence of 20% coarsely ground buckwheat bran, falling to ~ 11 in the presence of 20% fine buckwheat bran (Table 1). As pointed out in recent studies (Jazaeri et al., 2015; Quayson, Marti, Bonomi, Atwell, & Seetharaman, 2016), hydrophobic interactions are among the main forces involved in network formation in wheat dough. Therefore, the lack of exposed hydrophobic sites in the bran-containing dough samples may be indicative of an impairment of their extensional properties.

Since the addition of either type of buckwheat bran did not alter the protein profile in the systems under investigation (data not shown), it appears reasonable to attribute the effects previously discussed to the fact that proteins in the system did not undergo the structural rearrangements required to bring hydrophobic regions from the interior of the proteins (or of the protein aggregates) to their surface. These rearrangements largely depend on water availability and gluten hydration, as demonstrated by a number of spectroscopic solvation studies (Bonomi, Iametti, Mamone, & Ferranti, 2013; Bonomi et al., 2004) and they are independent of variation in lipid content (Huscka et al., 2012), which may derive from bran addition (0.8% increase in lipid content

in the dough at highest addition level of bran). As observed in Figure 1, common wheat proteins reached almost a peak in their structural arrangement at 45% water in the dough, corresponding to the optimal water absorption obtained from Farinograph analysis. In fact, at higher water level fluorescence emission seemed to reach a steady value. In the presence of buckwheat bran, gluten protein solvation was significantly reduced throughout the range of water content tested ($p<0.05$). In fact, proteins in doughs enriched with 20% of either coarse or fine bran did not complete their solvation (and therefore, the exposure of ANS-binding hydrophobic sites) even at water contents as high as 55%, as indicated by the continuous and progressive increase in fluorescence ($p<0.05$) (Figure 1). Nevertheless, these hydration values were incompatible with the formation of a dough but were associated to the production of a batter. These results suggest that the addition of buckwheat bran affects the partitioning of water in the dough limiting gluten solvation. In the presence of bran a complete hydration of gluten can be achieved at water levels incompatible with proper dough formation due to excessive protein dilution.

Figure 1 made also evident a more pronounced impairment of protein solvation when small-sized bran was used, which was significantly different at all water levels tested ($p<0.05$). Differences in the level of bran dispersion can affect locally the dynamics of gluten hydration. Consequently, it can be suggested that the higher dispersion of fine particles and a higher rate of hydration compared to the coarse one may account for reduced gluten hydration. Since gluten development is a dynamic process involving protein hydration as well as protein interactions induced by mixing, changes in hydration dynamics may well explain the observed differences in gluten structural arrangements. Together with gluten hydration, inhibition of gluten development by a chemical interaction mechanism involving ferulic acid has been indicated as one of the main causes for the detrimental effects of wheat bran addition to dough (Noort et al., 2010; Wang, Van Vliet, & Hamer, 2004). This hypothesis seems unlikely for the doughs used in this study due to the extremely low amounts of ferulic acid in buckwheat bran compared to wheat bran (Gallardo,

Jimenez, & Garcia-Conesa, 2006). On the other hand, inhibition by other chemical substances such as glutathione cannot be completely ruled out.

3.1.3 Thermo-mechanical behavior and phase transitions in dough during heating

The effect of enrichment in coarse or fine bran on the thermo-mechanical behavior of wheat dough was investigated by DMTA during a temperature sweep. This technique provides insights on the influence of phase transitions, e.g. starch gelatinization, on the mechanical properties of the dough at small deformations (Erickson et al., 2014). Figure 2A and 2B show the evolution of the storage modulus during heating of wheat doughs containing respectively coarse and fine buckwheat bran. In all samples G´ initially decreased going from 40°C to 50°C, approximately, due to the softening of the dough. In the temperature range between 50 and 60°C all dough samples showed a sharp increase in G´, which can be associated with the onset of starch gelatinization (Dreese, Faubion, & Hoseney, 1988; Jekle, Mühlberger, & Becker, 2016; Xie, Yu, Chen, & Li, 2008). In fact, the onset temperatures of starch gelatinization as derived from G´ (Table 1) were found to be highly correlated with those obtained by DSC analysis of the dough (R^2=0.879; p<0.00). This result confirms that the mechanical transition observed in the 50-60°C is the result of heat-induced gelatinization when the starch granule absorbs water and swells. Consequently, the further increase in G´ can be associated with the increased hydration of the starch granules and the gelling of the leached starch, reaching a maximum around 70-75°C, which can be associated with the peak gelatinization temperature T_{max}. At the peak temperature, the maximum gel strength G'_{max} is achieved, after which a typical decrease in the gel strength is observed with increasing temperature (Jekle et al., 2016).

Bran enrichment of wheat dough resulted in a progressive increase in the onset of starch gelatinization, which was significant only for the 10% and 20% level of inclusion of fine bran (Table 1)**.** Similarly, an increase in peak temperature was observed with inclusion of fine bran, which was significant at 20% addition (data not shown). Despite the presence of buckwheat starch in the bran, at 20% bran inclusion buckwheat starch was only about 5% of total starch. In excess

water, the ranges of T_{onset} for buckwheat and wheat starches are reported to be 51.5–62.3 and 51-60°C, respectively (Delcour & Hoseney, 2010; Noda et al., 1998). Therefore, it is unlikely that differences in gelatinization temperature between wheat and buckwheat starch can explain an increase in T_{onset} of 2.3°C with 20% fine bran inclusion. In order to explain the observed changes in gelatinization temperature it is essential to consider the main mechanisms which influence the melting process. According to thermodynamic theories describing the state diagram of starch in water mixtures (Van der Sman & Meinders, 2011), the starch gelatinization temperature is function of the volume fraction of water in the system (equation 1). Consequently, the applied variations in the amount of water added in the dough contribute in modulating the starch gelatinization process. However, this mechanism alone would not explain the observed increases in T_{onset} as extra water was added in the bran enriched doughs while the total starch content decreased, which should result in a progressive decrease in the onset temperature. As recently described by Jekle et al. (2016), starch gelatinization in the presence of other biopolymers such as gluten is modulated by a competitive hydration between the polymers. The addition of bran changes the partitioning of water in the system as it will compete with starch and gluten to absorb the available water. As described in equation (2), the partitioning of water is function of the volume fraction of bran, Φ_{bran}, and the specific water-bran interaction parameter χ. Sorption properties were similar for coarse and fine bran (supplementary material Figure S1). Therefore, the increasing level of bran alone can describe the changes in water partitioning in the dough, thus slowing down the hydration of starch. Consequently, it can be suggested that T_{onset} is modulated by both the volume fraction of bran, Φ_{bran}, and by the volume fraction of water Φ_{ws} potentially available for gelatinization (i.e. rescaled over starch volume fraction as described in equation 3). This hypothesis is confirmed by the results of the linear regression model shown in Figure 2C: the interplay between Φ_{ws} and Φ_{bran}, can well explain the observed variation in T_{onset} ($p<0.00$ for both Φ_{ws} and Φ_{bran}), following the equation:

$$T_{onset} = 111.9 - 94.1 \cdot \Phi_{ws} + 80.2 \cdot \Phi_{bran} \qquad (4)$$

According to the model, an increase in Φ_{ws} lowers T_{onset} while an increase in Φ_{bran} increases T_{onset}, which is in agreement with the physical mechanism described. Similarly, a good correlation was observed between the measured T_{max} and that predicted by a linear regression model with Φ_{ws} and Φ_{bran} (R^2=0.851; p<0.00 for both Φ_{ws} and Φ_{bran}; data not shown).

Variations in G'_{max} were also observed as function of bran addition level and size. In particular, the increasing addition of fine bran resulted in a progressive increase in G'_{max}. On the contrary, no clear trends could be observed with the addition of coarse bran. It should be noted that all fine bran enrichment level had a similar amount of water added to the dough while an increasing amount of water was added with progressive coarse bran enrichment of the dough. Following on the model proposed by Taylor & Bagley (1974, 1977), Steeneken (1989) demonstrated that the rheological properties of swollen starch granules in water suspensions are determined by the volume fraction occupied by the particles and by their rigidity. In diluted regimes, starch granules can swell to their maximum and the strength of the paste is mainly function of starch concentration. However, in concentrated regimes as is the case of wheat dough, starch granules cannot swell to their maximum and the amount of water becomes a limiting factor (Steeneken, 1989). Consequently, the starch rigidity has a key contribution to the paste strength. That is represented by a rigidity index of starch granules which provides the rate of increase in paste strength as function of starch concentration. It should be noted that the model of Taylor and Bagley applies to a binary water-starch system, where the variation in starch concentration reflects the variation in water to starch ratio. That is not the case of the complex dough formulation under study due to the addition of bran in replacement of flour and to the adjustments in water levels. In the dough systems under study, the rigidity of starch granules may be associated to the amount of water available for starch gelatinization. Therefore, it could be suggested that different trends observed between coarse and fine bran may be largely related to differences in granule rigidity and starch concentration consequent to the different water levels used.

The tan δ plots of wheat dough enriched in coarse (Figure 2A) and fine bran (Figure 2B) provided information on the contribution of the viscous and elastic modulus to the viscoelastic behavior during heating. The tan δ values for all samples were smaller than 1, suggesting that elastic properties predominated. However, a progressive inclusion in buckwheat bran – either coarse or fine bran – promoted an increase in tan δ with increasing level of bran enrichment (Figure 2 A,B), which was evident at the onset of starch gelatinization (Table 1). The increase in tan δ indicates an increase in the viscous behavior of the dough relative to the increase in elastic-like behavior. Until T_{onset}, the rheological behavior of the dough can be mainly related to the properties of the gluten network as starch and bran will mainly act as fillers. As previously discussed, gluten development is the result of a dynamic process in which the inclusion of bran affects the protein hydration mechanism. From this standpoint, the increase in tan δ with bran addition can be explained by the impairment in gluten development which was observed in the protein surface hydrophobicity study. This hypothesis is confirmed by the correlation between dough rheology, i.e. tan δ at onset, and gluten structural arrangements, i.e. F_{max}, (R^2=0.926; p<0.00).

3.2 Bread baking quality in relation to dough properties

The effect of buckwheat bran enrichment on bread quality is summarized in Table 2. All bran-enriched bread samples had lower specific volume than the control ($p \leq 0.05$), except for sample CB5 that showed no statistical differences from the wheat reference. At similar enrichment level, fine bran had always larger detrimental effects on bread specific volume than coarse bran ($p \leq 0.05$).

Although the detrimental effect of wheat bran on bread volume is well known and documented (Lai et al., 1989; Pomeranz, Shogren, Finney, & Bechtel, 1977), the effect of particle size on this parameter is still controversial: some authors demonstrated that enrichment in wheat fine bran had no effect on loaf volume (Curti, Carini, Bonacini, Tribuzio, & Vittadini, 2013; Sanz-Penella, Laparra, Sanz, & Haros, 2012). Coda et al. (2014) identified 160 μm as the optimal bran

particle size for bread production. Conversely, Noort et al. (2010) found a strong positive correlation between bran particle size and bread volume. The negative effects of bran were related to gluten quantity, as a strong relation was observed between bread volume and gluten yield. Aside from gluten yield, further insights on gluten structural arrangements and its relation with dough baking performance could provide valuable information on the influence of buckwheat bran on dough quality. To the best of our knowledge, these aspects have not been fully covered in the literature.

In our study, protein surface hydrophobicity experiments clearly pointed at a dependency of protein structural arrangement in the dough on both bran inclusion level as well as bran particle size. It can be suggested that gluten structural arrangement in the dough plays a key role in determining the bread baking performance. In fact, a progressive decrease in bread specific volume could be observed as function of the reduction in PSH index (Figure 3). As previously discussed, the changes in PSH index could be explained by incomplete hydration of gluten during mixing with increasing bran level and with reduction in particle size. Only with 5% addition of coarse bran, a reduction in PSH did not correspond to a significant change in specific volume. That may suggest a threshold in the structural arrangements of the gluten network beyond which negative effects on bread quality can be observed.

Crumb density significantly decreased with either coarse or fine bran enrichment, which was consequently related to the decrease in the specific volume of bread (R^2=0.914; $p< 0.00$). The addition of coarse bran resulted in a progressive increase in moisture content (Table 2). On the contrary, bread crumbs enriched with fine bran showed a higher moisture content compared to the wheat reference which was similar independently of addition level. The moisture content in the crumb was strongly correlated to the water content in the dough formulation (R^2=0.983; $p< 0.00$). Moisture content in the crumbs did not change during the 4 days storage in the sealed containers (data not shown).

3.3 Crumb texture

Bran addition resulted in detrimental effects on bread crumb texture, as hardness increased with a progressive increase in bran addition, while cohesiveness decreased (Table 2). At similar bran addition levels, the size of the effect was significantly different depending on the type of bran used. Springiness and resilience decreased in a similar manner with increasing bran addition (data not shown). In fact, cohesiveness was found to be highly correlated with both springiness as well as resilience (R^2=0.903, p<0.00 and R^2=0.908, p< 0.00, respectively; data not shown). The detrimental effect of bran addition on crumb texture, i.e. increased hardness and reduced cohesiveness, have been already reported in the literature by several authors (Heiniö et al., 2016; Hemdane, Langenaeken, et al., 2016; Lai et al., 1989; Pomeranz et al., 1977). However, a mechanistic elucidation of the physical contribution of bran on crumb texture is still missing.

In cellular solids, the perceived textural hardness of the material is related to its density, following on the Ashby-Gibson theory (Renzetti & Jurgens, 2016). In fact, a strong correlation between crumb hardness and crumb density was observed at day 0 (R^2=0.966, p<0.00). For such reason, the adapted Ashby-Gibson model was applied to correct for differences in crumb density (Figure 4A). Significant differences in corrected hardness could be observed among samples enriched in coarse bran and micronized bran at similar addition level. This result indicates that the changes in bread crumb texture can be only partially described by the density differences. Enrichment in fine bran clearly resulted into larger texture changes compared to the coarse one. Any deviation from the model can be ascribable to modification of the solid crumb structure around air cells as induced by the incorporation of bran. The interplay of several mechanisms can together account for the observed variations in corrected hardness. First of all, it should be noted that the significant variations in the volume fraction of water, Φ_{water} (Table 2), are likely to modulate the mechanical behaviour of the crumb. Additionally, the partitioning of water within the hydrophilic biopolymeric phase can also modulate its mechanical properties. Following on the thermodynamic theories previously described (equation 2), the partitioning of water in the crumbs under investigation will change with increasing volume fraction of bran Φ_{bran} Finally, an increase in the volume fraction of

bran may also enhance the elastic moduli of the crumb matrix, i.e. E_{film}, due to stronger particle interactions. Overall, the variations in Φ_{bran} and Φ_{water} can well account for all the described mechanisms. In fact, a linear regression analysis indicated that the interplay of these two parameters well describe the corrected hardness at day 0, as indicated by the high correlation between measured and predicted values (Figure 4B). The model equation derived from the analysis indicated that corrected hardness decreases with increasing Φ_{water}, while it increases with increasing Φ_{bran}, which is logic from a physical standpoint.

Aside from hardness, bran negatively affected the crumb ability to recover from the first compression, thus worsening its structural integrity when subjected to compressive forces. The decrease in cohesiveness (Table 2) is indicative of enhanced micro-fracturing of the solid lamellae around the air cells during the compression. It can be suggested that such micro-fractures can be enhanced by discontinuity in the polymeric crumb network resulting from increased volume fraction of solid particles, *i.e.* Φ_{bran} Furthermore, the influence of Φ_{bran} on moisture partitioning in the polymeric phase and the variations in Φ_{water} are also likely to contribute to the observed variations in cohesiveness. In fact, the interplay of Φ_{bran} and Φ_{water} could well describe the observed variations in cohesiveness, as indicated by the high correlation between measured and predicted values (Figure 4C). The model equation derived from the linear regression indicated that cohesiveness increased with increasing Φ_{water} while it decreased with increasing Φ_{bran}, which is in agreement with the described mechanisms.

During storage, a progressive increase in crumb hardness was observed for all breads as a result of staling (data not shown). The differences in hardness observed at day 0 among the wheat bread reference and the bran-enriched bread samples persisted during storage. It should be noted that the crumb samples were stored in sealed containers to distinguish the effects of starch retrogradation from moisture redistribution between crumb and crust. All hardness data were corrected for density by applying the Ashy-Gibson theory. The corrected hardness values were strongly correlated with the melting enthalpy of starch during storage (Figure 4D, $p < 0.00$). Hence,

the changes in hardness during storage for all bread types could be mainly related to the variations in density and starch retrogradation. Similarly, crumb cohesiveness during storage was inversely related to starch retrogradation (R^2=0.946, p<0.00).

4. Conclusions

Wheat bread enrichment with buckwheat bran resulted in altered rheological and baking properties compared to the reference white bread. In general, the deteriorating effects increased with progressive bran addition level and were larger with fine bran compared to the coarse one. The application of molecular and material science approaches provided a quantitative description of the physical mechanisms behind bran functionality in wheat dough. Briefly, the effect of buckwheat bran incorporation in wheat dough is related to water availability and water partitioning among the main polymeric components, i.e. gluten and starch. From a molecular standpoint, gluten surface hydrophobicity studies showed that increasing bran addition and reduction in bran size inhibited the development of a gluten secondary structure optimal for baking. On the contrary, the thermo-mechanical behavior of wheat dough during heating was found to be mainly a function of starch gelatinization. Within the conditions of this study, the onset of the gelatinization process was controlled by the interplay of water and bran volume fractions.

With regards to texture, variations in crumb hardness of freshly baked breads could be in part related to the variation in crumb density. However, an adapted Ashby-Gibson theory for cellular solids revealed that buckwheat bran inclusion induced significant changes in the mechanical properties of the solid crumb matrix that were modulated by variation in water and bran volume fraction. During storage, the mechanical properties of the crumb, i.e. corrected hardness and cohesiveness, were mainly function of starch retrogradation.

The insights generated suggest that the technological approaches to limit the negative effect of buckwheat bran inclusion in the dough should focus on counteracting the changes in water partioning in the dough as modulated by bran volume fraction, sorption properties and particle size.

Enzymatic and microbial fermentations are interesting technologies to achieve changes in the material properties of bran.

In the future, the physical mechanisms described in this study should be further investigated with brans from other sources, thus varying in composition (i.e. soluble and insoluble fractions), structure and sorption properties.

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Figure captions

Figure. 1. Intensity of ANS fluorescence at 470 nm as a function of water for the reference white flour (WF) and for flour enriched with 20% coarse (CB) and fine (FB) buckwheat bran. Excitation was at 390 nm.

Figure. 2. DMTA profiles for wheat dough enriched with varying levels of coarse (A) and fine (B) buckwheat bran (black lines: G´ modulus; red lines: tan δ). (C) Correlation between measured T_{onset} of starch gelatinization and the one predicted from Φ_{ws} and Φ_{bran} (p=0.00 for both Φ_{ws} and Φ_{bran}). Black lines in graphs C indicate 95% confidence intervals.

Figure 3. Observed variations in bread specific volume as function of the PSH index obtained in various bran enriched doughs which were varying in bran addition levels (5, 10 and 20%) and bran particle size (coarse vs. fine bran)

Figure. 4. (A) Measured crumb hardness of bran enriched breads (solid line) and hardness after correction for crumb density (dash line); black line: coarse bran (CB), red line: fine bran (FB). Interaction between type and % of bran is significant ($p \leq 0.05$). Different letters indicate significant differences for corrected hardness parameter (LSD; $p \leq 0.05$); (B) correlation between the corrected hardness measured at day 0 and the corrected hardness predicted from Φ_{water} and Φ_{bran} ($p < 0.00$ and $p < 0.02$ for Φ_{bran} and Φ_{water}, respectively): $Corrected\ hardness = 70.7 - 169.7 \cdot \Phi_{water} + 20 \cdot \Phi_{bran}$; (C) correlation between cohesiveness measured at day 0 and cohesiveness predicted from Φ_{water} and Φ_{bran} ($p < 0.00$ for both Φ_{water} and Φ_{bran}): $Cohesiveness = -3.8 + 11.6 \cdot \Phi_{water} - 1.3 \cdot \Phi_{bran}$; (D) Correlation between corrected crumb hardness and starch melting enthalpy during 4 days storage. Black lines in graphs B, C and D indicate 95% confidence intervals.

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Table 1. Characteristics of wheat doughs enriched with varying levels of coarse (CB) and fine (FB) buckwheat bran: water absorption from farinograph tests with corresponding dough water content, ANS binding parameters and DMTA parameters. All data are expressed as the mean ± SD (n=3 from independent doughs). Different letters in the same column indicate statistically significant differences (LSD; $p \leq 0.05$)

	Water absorption properties		ANS binding parameters[c]			DMTA parameters		
Samples	Water absorption[a] (Baker's %)	Dough water content[b] (%wb)	F_{max}[d]	K_d^{app} (μmol ANS/g flour)	Protein surface hydrophobicity index (PSH)[e]	T_{onset} starch gelatinization (°C)	G´$_{max}$ (Pa)	Tan δ at T_{onset}
WF	57.4	45.4	68±1.2^{a}	0.344±0.012^{e}	196	54.2±0.2^{c}	88711±3548^{bc}	0.388±0.004^{d}
CB5	58.6	45.8	51±3.1^{b}	0.399±0.022^{d}	128	54.9±0.3^{bc}	85991±3004^{c}	0.407±0.020^{bcd}
CB10	59.6	46.1	39±0.7^{c}	0.460±0.042^{c}	84	54.8±0.2^{bc}	93383±7900^{abc}	0.412±0.014^{abc}
CB20	61.6	46.8	21±0.5^{e}	0.468±0.032^{c}	44	55.3±0.1^{abc}	88517±1819^{bc}	0.418±0.003^{ab}
FB5	58.2	45.6	40±1.8^{c}	0.525±0.021^{b}	77	54.9±0.5^{bc}	93751±6406^{abc}	0.406±0.023^{bcd}

FB10		58.0	45.6		32±2.2[d]	0.908±0.061[a]	35		55.7±1.3[a b]	95989±6962[ab]	0.412±0.007[abc]
FB20		58.1	45.6		12±0.5[f]	1.065±0.088[a]	11		56.5±1.2[a]	101689±4387[a]	0.429±0.011[a]

[a]Water absorption from farinograph tests

[b]Dough water content calculated considering the initial moisture of the flour/mixtures and the water added based on Farinograph analysis

[c]ANS binding parameters were calculated from ANS-titration experiments analyzed through Scatchard plots (n=2 for each ANS concentration)

[d]Fluorescence intensity at saturating ANS concentration corrected for the protein content

[e]PSH index defined as: $F_{max} \cdot (K_d^{app})^{-1}$

Table 2. Characteristics of wheat bread and bread crumbs enriched in coarse (CB) and fine (FB) buckwheat bran, including volume fractions of water (Φ_{water}) and bran (Φ_{bran}) calculated based on crumb composition. Data are reported as mean ± SD (n=4 for specific volume and moisture content; n=12 for crumb density, hardness and cohesiveness). Different letters in the same column indicate statistically significant differences (LSD; $p \leq 0.05$).

Sample	Specific volume	Crumb Density	Moisture content	Hardness	Cohesiveness	Φ_{water}	Φ_{bran}
	(mL/g)	(g/mL)	(%)	(N)			
WF	3.5 ± 0.6^{a}	0.23 ± 0.01^{d}	43.7 ± 0.1^{e}	1.2 ± 0.1^{e}	0.88 ± 0.01^{a}	0.406	0.000
CB 5	3.6 ± 0.2^{a}	0.22 ± 0.01^{d}	44.3 ± 0.1^{c}	1.1 ± 0.1^{e}	0.88 ± 0.09^{a}	0.409	0.029
CB 10	3.3 ± 0.2^{b}	0.25 ± 0.01^{c}	44.6 ± 0.2^{b}	1.5 ± 0.1^{d}	0.87 ± 0.02^{ab}	0.410	0.058
CB 20	2.7 ± 0.2^{c}	0.31 ± 0.02^{b}	45.5 ± 0.1^{a}	3.0 ± 0.3^{c}	0.83 ± 0.01^{c}	0.415	0.116
FB 5	3.3 ± 0.2^{b}	0.25 ± 0.01^{c}	44.2 ± 0.1^{cd}	1.7 ± 0.1^{d}	0.86 ± 0.01^{b}	0.408	0.030
FB 10	2.6 ± 0.1^{c}	0.30 ± 0.02^{b}	44.2 ± 0.1^{cd}	3.6 ± 0.6^{b}	0.82 ± 0.02^{c}	0.408	0.059
FB 20	2.3 ± 0.1^{d}	0.40 ± 0.01^{d}	44.1 ± 0.1^{d}	7.3 ± 0.4^{a}	0.75 ± 0.01^{d}	0.408	0.118

Fig. 1

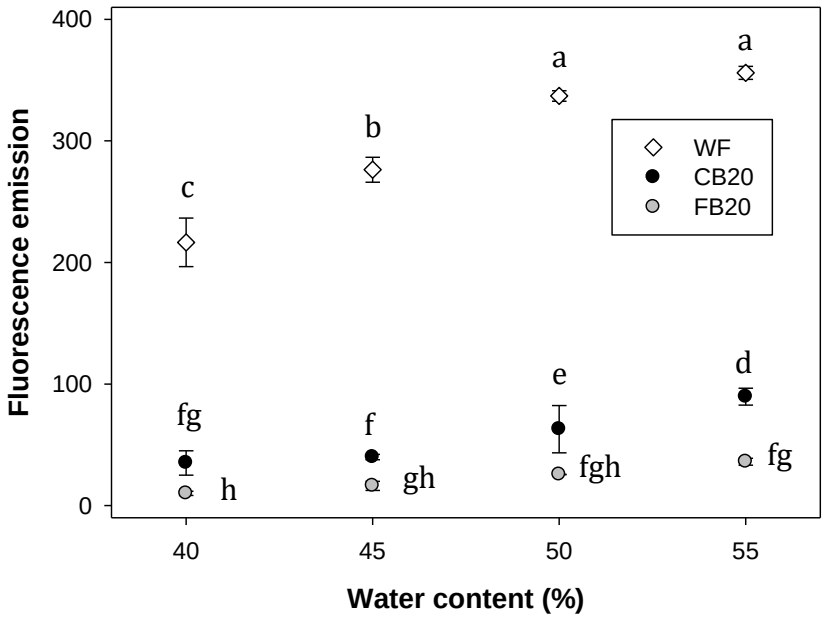


Fig. 2

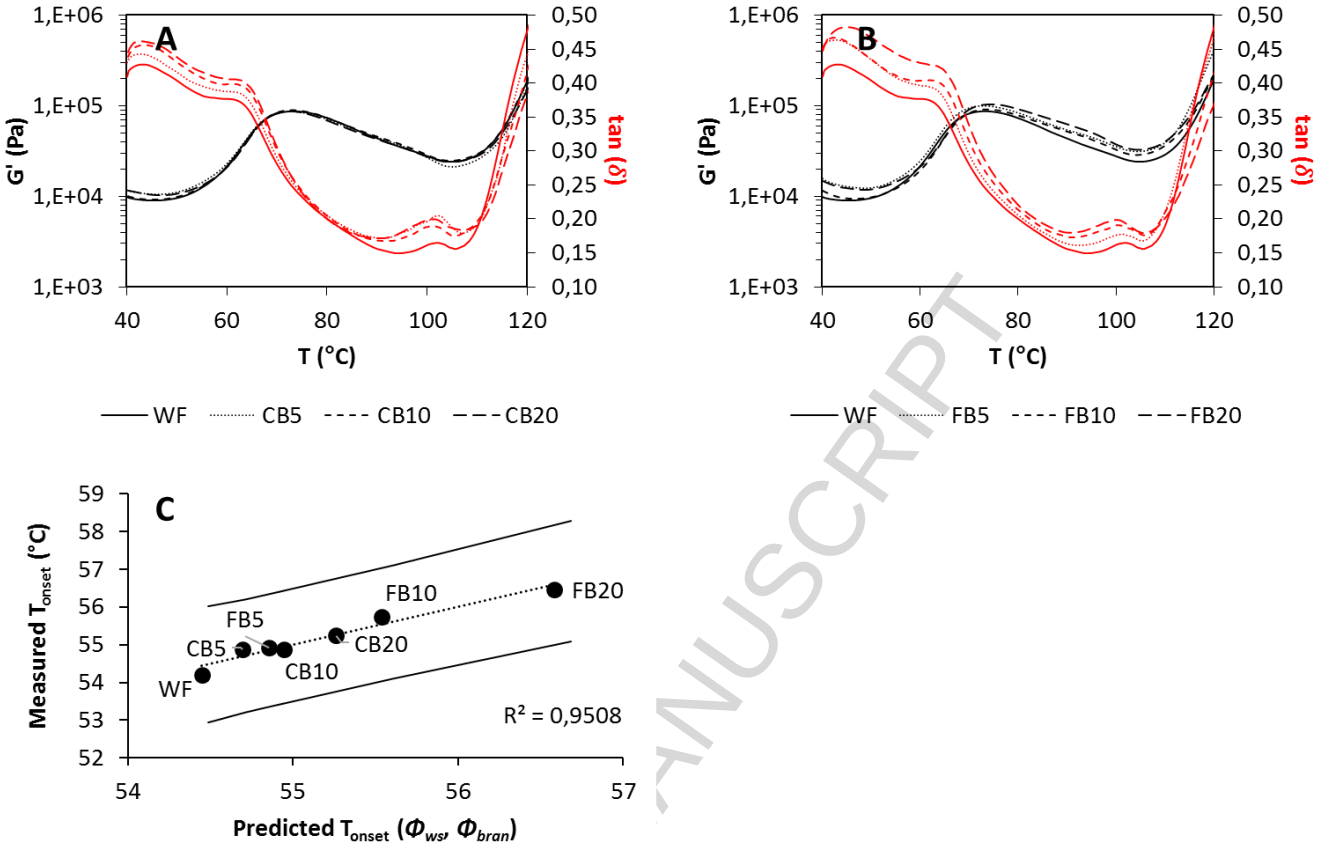


Fig. 3

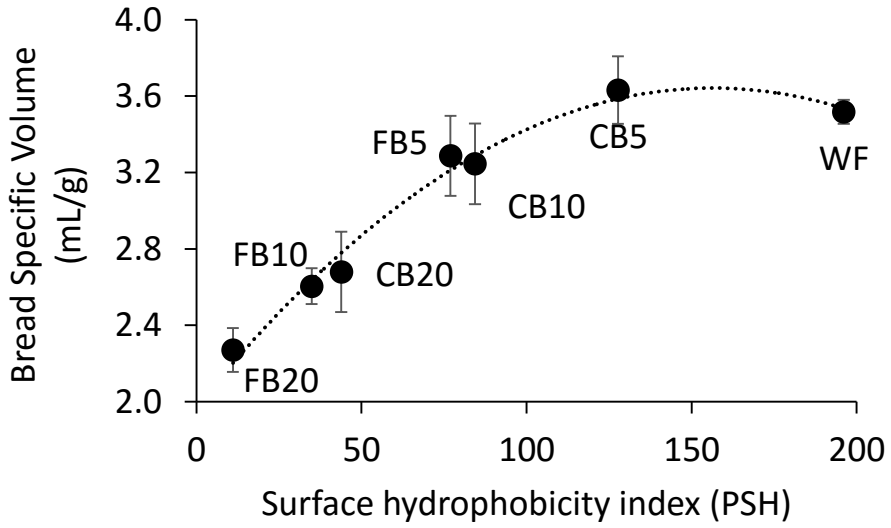
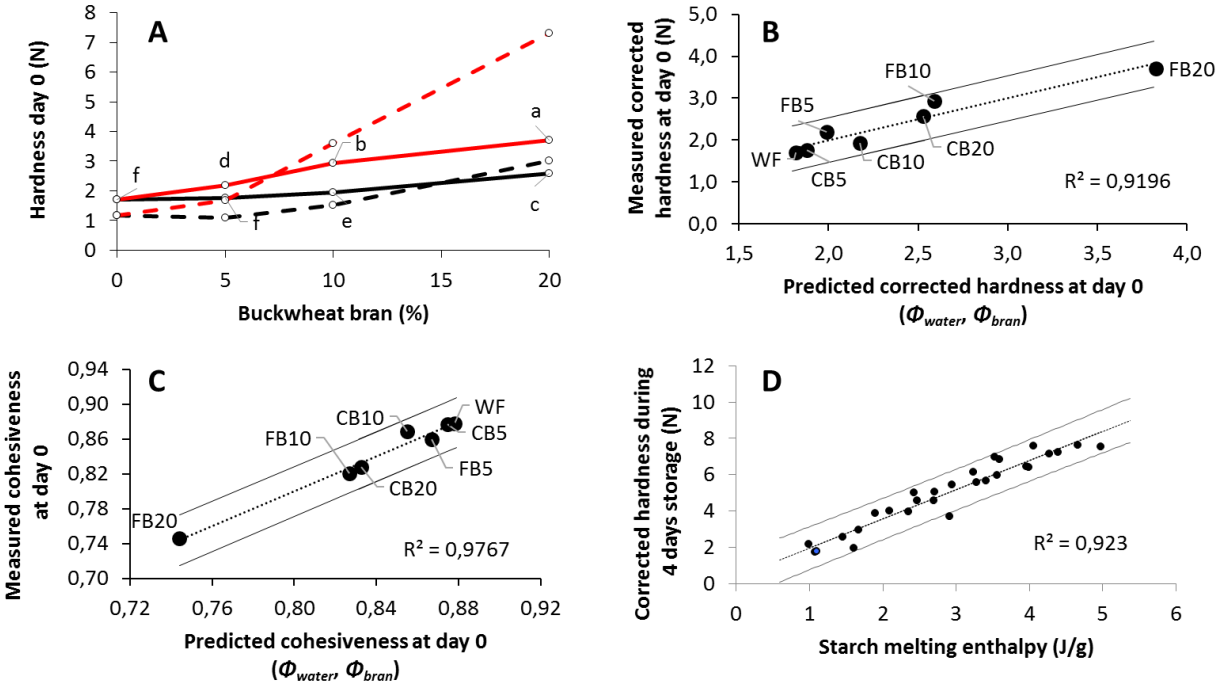


Fig. 4



Graphical abstract