THERMO-MECHANICALLY INDUCED PROTEIN AGGREGATION AND STARCH STRUCTURAL CHANGES IN WHEAT FLOUR DOUGH

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Running head: Proteins and starch under thermo-mechanical constraints

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Abstract

Various studies have been carried out on wheat flour to understand protein and starch changes when subjected to mixing and temperature constraints, but structural changes of proteins and starch at the typical moisture levels of a dough system, are not fully understood. The aim of this research was to improve our understanding of (micro) structural changes at the mesoscopic level, using: empirical rheology, microscopy (light and scanning electron microscopy), sequential protein extractions and glutenin macro-polymer (GMP) wet weight, along mixing-heating-cooling stages of the Mixolab® assay. Studies were performed in three wheat flours with different protein content. The rheological analysis allowed identifying the role of the proteins and the relationship between the protein content and different primary and secondary parameters obtained from the recorded curves. The progressive heating-mixing stages during the Mixolab assay, results in a dynamic re-and de-structuring of proteins involving interactions between the flour proteins from water-soluble, to SDS soluble to SDS insoluble and vice-versa. The microstructure analysis using light, polarized and scanning electron microscopy revealed the changes that proteins and starch molecules undergo during mixing, heating and cooling. Qualitatively the starch structural changes, swelling and gelatinization observed by microscopic techniques, shows some parallels with protein (and glutenin) content of the respective flour. Nevertheless, this tentative finding needs further confirmation by studying flour samples with a large difference in glutenin content.

Key words: wheat dough, Mixolab, proteins, rheology, microstructure

Introduction

Wheat flour dough has unique rheological properties, making it very suitable for bread-making (Bushuk 1998). Breadmaking is a dynamic process where several physical and chemical changes are involved (Rosell 2011). The gluten proteins are largely responsible for the rheology of wheat flour dough, structural formation during mixing, and gas-holding, whereas the role of starch is mainly implicated at final textural properties and product stability after baking. In fact, recently Lagrain et al. (2012) confirmed by crumb compressive tests, image analysis and ultrasonic inspection, that when keeping starch properties or moisture content, gluten properties determine bread crumb density and its foam structure without affecting the rheological properties of the crumb cell walls, and starch role is a major determinant of the elastic modulus of bread crumb increase upon storage. Gluten consists of the monomeric gliadins and the more complex glutenins. Glutenin consists of high and low molecular weight glutenin subunits (HMWGS and LMWGS), that are linked together by disulphide bonds (Shewry 1992). Since the paper of Ewart (1968), various molecular structures have been proposed for glutenin. Thus far, there is no consensus on the molecular / polymer structure of glutenin that can explain rheological properties from a molecular structure to macroscopic functionality model. It is also difficult to link molecular information on SH-SS with dough properties. Free SH groups have been reported in the range of 2-4 µmol/g dough (Andrews et al. 1995), but still it has not been possible to pinpoint the 'rheologically effective disulphide bonds' from the 'rheologically in-effective disulphide bonds' (Bloksma, 1972). Furthermore, later it has been shown that when doughs are mixed with SH-blocker NEMI, the rested doughs can have the same rheological response as the reference dough without NEMI (Don, 2009). It was revealed that non-covalent interactions of mesoscopic glutenin aggregates can rheologically

compensate for covalent interactions. Therefore we focus here on a level between the molecular scale (~10^{-3} µm) and visible with the unaided eye macro scale (~10^{3} µm and over): the mesoscopic scale (~10^{-1} – 10^{2} µm). This concept of mesoscopic glutenin particles, has been shown to be a relatively new element to improve our understanding of factors affecting wheat flour dough properties (Don et al., 2003, 2005).

Dough mixing is a key step in wheat flour processing, but during mixing a sequence of events takes place: 1) Mixing of flour and water with the help of mechanical energy input leading to distribution of flour components 2) hydration of flour particles, favouring both non-covalent but also covalent interactions 3) finally yielding the formation of a continuous visco-elastic network structure (Cuq et al., 2003).

Assessing the rheological properties of wheat flour dough with a recording mixer, is a common physico-analytical practice, both in scientific research as well as in routine analysis (Rosell and Collar, 2009). It already has been established that all rheological tests on dough, whether fundamental or empirical, give useful information to predict the end-use quality of wheat flour. Clearly, the best predictions can be expected when the rates and the extent of the deformation are in the same range as those during dough processing (Dobraszczyk and Morgenstern, 2003). Small deformation rheology is sensitive to starch–starch, starch–protein and protein–protein interactions (Rosell and Foegeding, 2007), but only large deformation measurements can provide information about the extent of the contribution of long-range (protein–protein) and short range (starch–starch and starch–protein) interactions to the viscoelastic behaviour of wheat flour dough (Amemiya and Menjivar, 1992)

Graveland et al. (1982) established the fractionation procedure based on wheat protein solubility / insolubility in 1.5% SDS. Aqueous SDS solution is regarded as one of the

most efficient solvents for separating extractable and un-extractable gluten proteins under unreduced conditions Singh et al. (1990). The quantity of the so-called SDS insoluble glutenin fraction is significantly correlated with: dough development time, dough strength and bread loaf volume (Weegels et al., 1996; 1997). The mixing studies by Don et al. (2003; 2005) reflect glutenin aggregation changes at constant temperature dough processing and handling (T = 30°C). Next to the physico-mechanical effect of mixing on glutenin aggregate size, the effect of elevated temperatures is expected to change the aggregated state of the gluten proteins. Elevating dough mixing temperatures will swell wheat starch granules, raising the viscosity of the dough (dough pasting) (Rosell et al., 2007), and pasting properties have been revealed as useful predictors of bread firming behaviour during storage (Collar, 2003).

In a Mixolab® assay the effects of both mixing and heating on wheat gluten proteins and wheat starch can be noticed as a torque reading vs. a time-temperature axis. The test sample remains doughy throughout the measurement, keeping moisture at similar levels as in bread dough. Structural changes of proteins and starch at the typical moisture levels, mixing-time and temperature/pasting regimes of a Mixolab® assay, are far from clear. Therefore, the aim of this study was to reveal structural changes at the mesoscopic level, using: microscopy -Light Microscopy (LM) and Scanning Electron Microscopy (SEM), sequential protein extractions and glutenin macro-polymer (GMP) wet weight, along mixing-heating-cooling stages of the Mixolab® assay. The study will be focussed on the matrix states around, and at the C1 (peak), C2, C3, C4 and C5 Mixolab® readings. For this purpose, three different flours were selected on basis of protein quantity and C1 mixing time: Corde Noire, Gruau Rouge, Ficelle Verte.

Materials and Methods

Wheat flour characterization

Three commercial flours from soft wheat were provided by Chopin Technologies (Villeneuve-la-Garenne Cedex, France), which are commercially named as Gruau Rouge, Ficelle Verte and Corde Noire. Flours were characterized for moisture, protein, fat and ash content following the ICC standard methods (1999). Total carbohydrates were determinate by difference. Damage starch was determined according to the ICC Standard method n°172 (ICC, 2011). Flour alveograph parameters were determined according to ICC Standard method n°121 (ICC, 2011).

Mixolab® analysis

Wheat flour was poured in the Mixolab® bowl and mixed with the necessary amount of water for reaching optimum dough development (ICC, 2011). Wheat dough weight was fixed to 75 grams. The Mixolab® profile carried out in order to characterize dough consistency changes due to dual mixing and temperature constraint starts at 30°C and with constant mixing speed of 75 rpm. Dough mixing was carried out at 30ºC for eight minutes and then the temperature was increased up to 90°C over 15 min at the rate of 4 °C/min. Bowl temperature was held at 90°C for 7 min, then cooled to 50°C over 10 min at the rate of 4°C/min and finally held at 50°C for 5 min. The duration of each assay was 45 minutes. Figure 1 shows a typical curve recorded in the Mixolab® along the different stages (mixing, heating, cooling). Detailed description of the physical changes that occurred along Mixolab® measurement was reported by Rosell et al. (2006). Briefly, the first part of the Mixolab® curve records the dough behavior during mixing and overmixing; during this stage, the torque increased until it reaches a maximum (C1). At that point, the dough is able to resist the deformation for certain time, which determines the dough stability. The simultaneous mechanical shear stress and

146 temperature constraint (2nd stage) decrease the torque until a minimum value (C2) that
147 could be related to the beginning of the protein structure destabilization or protein
148 weakening. As the temperature increases starch gelatinization takes place (3rd stage)
149 with a concomitant increase in the torque until a new maximum value (C3). A reduction
150 in viscosity is observed in the 4th stage derived from the physical breakdown of the
151 starch granules, leading to a minimum value of the torque (C4). The decrease in the
152 temperature produces an enhancement in the dough consistency (stage 5th), resulting in
153 a maximum torque (C5). Parameters obtained from the recorded curve are detailed in
154 Table 1. In addition, the slopes defined along ascending and descending curves were
155 calculated. Values reported in Table 2 are the average of ten measurements.
156
157 **Dough sample preparation for LM and SEM survey and analysis**
158 After recording the times where main changes occur (C1, C2, C3, C4, C5), assays were
159 repeated stopping the analysis at each stage. Sampling for microstructure studies is
160 detailed in Table 1. Dough samples were quickly transferred to a freezer and then
161 subjected to freeze-drying.
162
163 ***Light microscopy (LM)***
164 Flours and freeze-dried doughs were suspended in distilled water (8% w/w) and kept
165 vortexing till use. The suspension was poured and spread out onto microscope slide and
166 samples were dehydrated using pure ethanol followed by acetone and finally air.
167 Samples were either directly observed under both light and polarizing optics or stained
168 with specific dyeing reagents. Starch was detected using iodine solution, and proteins
169 were detected with Ponceau Red. The dried samples were stained with iodine solution
170 (0.2 % w/v iodine and 2 % w/v potassium iodate) for 10 min and then with Ponceau 2R

(0.2 % w/v Ponceau 2R in 50 % ethanol containing 0.18 % v/v of 0.5M H_2SO_4) solution for 10 min. After staining, sections were rinsed in distilled water, followed by a wash in 70% (v/v) ethanol, absolute ethanol, acetone and finally air drying. Samples were mounted in fluorescence-free immersion oil and viewed directly. The distribution of protein and starch in the sample was observed using a light/fluorescence Nikon Eclipse 90i microscope (IZASA, Madrid, Spain). Proteins appeared red whereas starch appeared blue. Sections were photographed using a Digital Sight DS-5Mc color camera (Nikon Instruments Europe BV, Amsterdam, The Netherlands). Digital images were captured directly to the computer from three to five regions of the sample surface. Reported images were chosen to best represent the set of sample images obtained.

Scanning electron microscopy (SEM)

Flour and freeze-dried dough samples were mounted on metal stubs using double sided stick tape and sputter-coated with 100–200 Å thick layer of gold and palladium by Ion Sputter (Bio-Rad SC-500, Aname, Madrid, Spain). Analysis of the specimens was performed at 10 kV accelerating voltage with a scanning electron microscope (S-4100, Hitachi, Ibaraki, Japan) equipped with a field emission gun, a back-secondary electron detector and an EMIP 3.0 image data acquisition system (Rontec, Normanton, UK) from the SCSIE Department of the University of Valencia.

Dough samples for soluble/insoluble protein analyses

The numbers in Figure 1 show the parts of the Mixolab® analyses where a sample has been taken for soluble/insoluble protein analyses. Table 1 shows the sample numbers that have been analyzed for protein extractability. Sampling after certain mixing times went as follows: 1) Stop the Mixolab®, 2) Collect the dough sample as quickly as

196 possible, 3) Freeze the dough sample in liquid nitrogen, 4) Lyophilise the collected
197 samples and 5) Powder the sample on a Retsch mill for the extractability study.
198
199 **SDS insoluble gel-proteins (GMP) and soluble proteins**
200 For the determination of the 1.5% SDS soluble proteins and wet-weight of the 1.5%
201 SDS insoluble proteins an adapted sequential extraction method was used, largely based
202 on the original extraction procedure of Graveland et al. (1982). Weigh 100 mg of flour
203 or powdered dough in an Eppendorf tube (2mL). A pre-extraction of water-solubles was
204 done with ~2mL of 1% NaCl solution then centrifuged for 10 minutes at 10,000 rpm
205 (Eppendorf), the supernatants were collected. Then 1.8 mL of demineralised water was
206 added and vortexed vigorously; add 200µL of 15% SDS solution to the suspension and
207 shake gently to disperse flour and dilute SDS (1.5%). Centrifuge the tubes for 30
208 minutes in an Eppendorf centrifuge at 10,000 rpm. The relative protein content of the
209 supernatants was determined by BCA method (SERVA Electrophoresis,
210 Raamsdonksveer, The Netherlands), taking flour as index 100%. For processed dough
211 the starch phase is known to swell more, although most of the starch remains insoluble
212 in cold water or 1.5% SDS. The 1.5% SDS insoluble proteins remain on top of the
213 starchy phase. The 1.5% SDS insoluble proteins are rendered soluble by reduction in
214 1.5mL of 1.5% SDS with 0.2% DTT. The Eppendorf tubes are centrifuged (30', 10,000
215 rpm) after which the supernatant is poured off. The remaining starchy gel is weighed
216 and subtracted from the gel-weight (starch + disulphide linked GMP-gel proteins) of the
217 previous centrifugation under unreduced conditions. This provides a wet weight
218 estimation of disulphide linked SDS insoluble gel-proteins, also called Glutenin Macro
219 Polymer (GMP). The extractions were done in triplicate.
220

Statistical analysis

Experimental data were statistically analyzed by using Statgraphics V.7.1 program (Bitstream, Cambridge, MA, USA) to determine significant differences among them. Fisher's least significant difference (LSD) procedure was used to discriminate among the means at the 95.0% confidence level.

RESULTS AND DISCUSSION

Mixing and thermal behaviour of wheat flours

The behaviour during mixing and heating of three commercial flours, selected due to their different protein content, were determined using the Mixolab® (Figure 1). Wheat flours differed in their protein, ash, damaged starch content and their Alveograph parameters (P, L, W, P/L) (Table 2). Primary and secondary parameters defined from the Mixolab® plots are listed in Table 2. For comparing purposes, analysis of the different flour behaviour was carried out at constant consistency (C1 of 1.1 Nm), where hydration was not the constraint. Primary and secondary Mixolab® parameters were significantly dependent on the type of flour, with exception of amplitude, temperature at C3, pasting temperature range and the delta slope (related to the speed of amylose retrogradation during cooling). Time to reach the maximum dough development (C1) and dough stability during mixing were significantly dependent on the amount of protein of the wheat flour, being shorter or lower with the flour of lower amount of protein, respectively. Proteins, besides damaged starch and arabinoxylans, are the main components involved in water adsorption and dough hydration, although proteins due to their major abundance are of great importance as revealed by the present results for water absorption (Table 2). In addition, protein nature is also important, if exogenous

246 proteins are added, determines the development time or time necessary for hydrating all
247 the compounds (Bonet et al., 2006). Dough stability related to the strength of the protein
248 network was significantly higher with higher protein content flours, which was also
249 reflected in the Alveograph parameters. No significant differences were observed on the
250 amplitude, parameter associated to dough elasticity (Rosell and Collar, 2008). The
251 parameter associated to protein weakening (C2) showed the highest value with the
252 highest protein content flour. The flour with the lowest content of proteins had the C2 at
253 the lowest temperature. The combined effect of the mechanical shear stress and the
254 temperature constraint produced a decrease in the torque that has been related with the
255 beginning of the protein destabilization and unfolding (Rosell et al., 2007). In wheat
256 flours, the minimum torque (C2) has been detected in the range 52–58 ºC, further
257 protein changes during heating might be masked by the modification of the physico-
258 chemical properties of the starch (Rosell et al., 2007). Regarding the starch the wheat
259 flour with the lowest protein content showed the highest consistency after starch
260 gelatinization (C3), and also the highest stability during heating (C4). This finding
261 agrees with previous results of Symons and Brennan (2004) describing a relationship
262 between the peak viscosity and the starch content and its degree of swelling.
263 No strong relationship was found between the protein content of the flours and the
264 proteins weakening range, but we can highlight a few results. It was observed that there
265 are significant differences in protein weakening (C2) between the highest protein
266 content flour and the others. Specifically, C2 of Gruau Rouge was 0.55 Nm, whereas for
267 Ficelle Verte and Corde Noire ranged 0.44-0.41 Nm, and they showed significant
268 differences in their protein content (Gruau Rouge 14.9%, Ficelle Verte 9.9%, and Corde
269 Noire 10.96%). In addition, at C4 the values for Gruau Rouge vs Ficelle Verte and
270 Corde Noire were far apart: 1.61Nm vs. 2.02 and 1.93 Nm. The starch gelatinization

range was inversely related to protein and directly related to the carbohydrates content. The flour with the highest protein content showed the greatest gelling, in which the amylose chains which leached outside the starch granules during the heating, are prompted to recrystalize. The re-association between the starch molecules, especially amylose, results in the formation of a gel structure. This stage is related to the retrogradation and reordering of the starch molecules and low values of setback indicates low rate of amylose retrogradation and low syneresis (Rojas et al., 1999).

Studies performed with wheat dough containing different hydrocolloid combinations indicated that the overall effect on the mechanical shearing and thermal treatment of the wheat dough can be studied using the different slopes defined in the Mixolab® plots (Bonet et al., 2006). The parameter α described the effect of the combination of mechanical shearing and slight thermal treatment on the wheat dough. Whereas the parameters β, γ, and δ indicated the behaviour of wheat dough during heating, holding at 90°C, and cooling, respectively, they were thus mainly associated with starch changes. The protein weakening occurred faster in the flour with the highest protein content. The rates associated to starch changes were faster in the wheat flour with the highest protein content. Starch gelatinization rate and gelling was slower in the flours with lower protein content. The damage starch did not show a significant contribution to dough absorption and only a significant effect was detected when temperature increased (during protein weakening range).

SDS insoluble gel-proteins (GMP), the SDS soluble proteins and water-soluble proteins in relation with the Mixolab assay

The GMP-gel wet weight per gram flour of the three flour samples Gruau Rouge, Corde Noire and Ficelle Verte were respectively: 3.4 ± 0.1, 2.7±0.1 and 1.7±0.1 g/g. These

differences in GMP-gel wet weight run in parallel with respective flour protein content and mixing times to peak C1. Taking the initial values of the respective flour as 100%, the protein extractions for the flour samples can be plotted in a single figure against the respective sample numbers and average dough temperatures (Figure 2a-c). The mixolab torque (Tq) vs sample number is also given in Figure 2 (2d). Going from flour towards dough peak (C1 at 30°C) it can be observed that the initially SDS insoluble gel-proteins are rendered soluble in SDS by the mixing action. This is in agreement with earlier observations (Weegels et al. 1996, Don et al. 2003). After this dough mixing step the average dough temperature is increased, resulting in a progressive re-aggregation of apparently disulphide linked SDS insoluble proteins. It is perhaps remarkable that the heat induced re-aggregation of GMP seems to start at such a low average dough temperature (36°C, sample #2). Andrews et al. (1995) report somewhat higher temperatures for significant loss of free SH > 50°C, although some loss of relative free –SH can be observed already around 40°C. We suspect that it was too difficult to significantly detect the losses of free SH along the temperature range 50°C > T > 30°C. Physical accessibility of SH groups (in the µmol range and even less) can be affected, because our results show (Figure 2a) that glutenin apparently already starts aggregating into SDS insoluble structures between 35 – 45°C. About 50-80% recovery can be noticed due to a mild temperature induced aggregation. This also shows that our choice to focus at the mesoscopic level of SDS insoluble GMP re-aggregation provides new information. Furthermore, it reveals that separating fractions on basis of SDS solubility is an effective way for studying the re- and de-structuring of key protein fractions in processed dough. On the level of the instrument we should keep in mind that the mixing bowl surface temperature can be higher. We calculated this difference for the #1-#4 sampling points and found that the average bowl temperature is ~3°C higher than

the average temperature measured by the probe. Therefore, the temperature of dough in direct contact with the bowl surface is higher, but remained < 50°C at point #3 (fig 2a) where recoveries of SDS insolubles are noted between 70-90% (Ficelle Verte 88%, Corde Noire 76%, Gruau Rouge 70%). The recovery percentages of GMP from #1 to #3 in Figure 2a show that Ficelle Verte had the highest recovery rate, Corde Noire intermediate and Gruau Rouge the slowest recovery. Figure 2b shows a steep decrease of SDS soluble protein for Ficelle Verte (90%) compared to Corde Noire and Gruau Rouge at point #3 (resp. 120%. 116%). These differences in aggregation can be explained from our extraction data (2a-b) and the dough consistency (2d) as follows:

1) The rates of dispersing the insoluble wheat proteins with a low protein quality Ficelle Verte (lowest flour GMP, shortest C1-time), intermediate quality Corde Noire (intermediate flour GMP, intermediate C1-time) and high quality Gruau Rouge (highest flour GMP, highest C1-times) lays down the path for a faster heat-induced re-aggregation of GMP after C1-time when dough is warmed-up. A better distribution of protein aggregates in dough (SDS soluble, but not water-soluble) can re-assemble more effectively than less well-dispersed proteins.
2) The measured consistency of the warm doughs at sampling points #2 and #3 show torques for Ficelle Verte <Corde Noir <Gruau Rouge (Figure 2d) in compliance with respective flour GMP levels, hence the respective initial re-aggregation rate into SDS-insolubles at mild heating, can be related to the respective dough consistency. It is very likely that aggregation in a lower consitency environment will tend to run faster (low Tq FV) than in a higher consistency medium (higher Tq, GR).

For dough samples taken at Mixolab® stages #4(C2) and #5 the status of SDS insoluble gel-proteins hovers somewhat under (#4=C2) and over (#5) the 80% recovery mark. This indicates that mixing forces that are known to disrupt glutenin aggregates (Don et al. 2005) are competing with heat-induced re-aggregation. The C2 point coincides with a minimum in the Mixolab® curve, it is well-possible that over-mixing combined with heat-induced re-aggregation results in a more discontinuous gluten network with a lower resistance to movement, hence the minimum in the observed torque (Nm). When dough heating proceeds (#6, #7), the heat induced aggregation of gluten(in) proteins apparently overruns the disruption by mixing, resulting in recoveries of about 100% and over (120%) the initial flour GMP wet-weight. The fact that the SDS insoluble quantity exceeds the level of the flour reference indicates that also other proteins fractions may have ´co-aggregated´ with the insoluble glutenins. At the final stage water-holding of the SDS insoluble gel proteins is compromised (lower recovery), this shows that prolonged heating brings gluten proteins to a more denatured aggregated state.

Figure 2b shows the results for the SDS soluble proteins (SDSS). For all three flour samples the initial mixing stage to C1 (#1) renders the glutenin proteins soluble, as shown by Don et al. (2003). When heating and mixing proceeds (#2, #3, #4=C2) the SDS soluble proteins are further re-aggregated into SDS insoluble structures as indicated by the increase in GMP-gel proteins in Figure 2a. During further mixing and heating (samples #5, #6=C3,#7=C4, #8=C5) the recoveries of SDS soluble proteins are between 95 – 110%. There is not a fully clear parallel between the recovery levels of SDS soluble (Figure 2b) and GMP-gel (Figure 2a). Specifically at point #6 Figure 2a shows that the GMP is 110-120%, SDS soluble fraction > 100%, but there is a loss of water-soluble proteins ~80% recovery (Figure 2c). Tentatively, the progressive heating-mixing stages during the Mixolab assay, results in a dynamic re-and de-structuring of

proteins involving interactions between the flour proteins from water-soluble, to SDS soluble to SDS insoluble and vice-versa. This has been suggested earlier by Schofield et al. (1983) for heated gluten. Later on, Rosell & Foegeding (2005) also confirmed that hypothesis by studying the viscoelastic properties of gluten subjected to heating-cooling cycles. In that study, the storage modulus of the gluten proteins underwent a progressive decrease with the temperature increase that has been associated to protein unfolding. In a more molecularly oriented gluten study, the proteins showed a minimum value of storage modulus (G') at 57ºC, indicating a thermal transition derived from the protein crosslinking involving SH/SS interchange, oxidation and hydrophobic interactions (Li & Lee, 1998). The SH/SS interchange is an interesting notion, but here we will focus on the meso- and macro scale, but it is clear that when dissolving GMP, the DTT reduces the mesoscopic heat aggregated glutenin protein structures completely into subunits soluble in 1.5% SDS. As with free SH measurements it is doubtful whether complex macroscopic phenomena can be explained with measurements down to the molecular level of glutenin subunits.

Figure 2c shows that the result for the water-extractability of proteins (albumins and globulins) vs. the mixing-heating steps of the Mixolab® assay. For C1 the results clearly show an increase in water-soluble proteins for all three flour samples. As mixing and heating progresses (#2, #3, #4=C2, #5, #6, #7 and #8) the relative recovery of water-extractable proteins decreases from 100% towards about 75% at the final stages (#7=C4 and # 8=C5). There are some minor differences in aggregation rate of water soluble proteins, between the three flour samples; the overall picture is that water-solubility is compromised. Looking at results at #6 it is plausible that unrecoverable albumins and globulins ´co-aggregated´ into one of the water-insoluble fractions.

Especially into the SDS insoluble part when heating is > 70°C resulting in recoveries > 120% for GMP. Clearly albumins and globulins are a minor fraction of the wheat flour proteins, and 20% of this minor fraction represents even less. However, the role of water-soluble protein has been disregarded in comparison to gluten proteins; it is interesting to see in this study revealed that it becomes part of the water-insoluble fraction when processed.

Protein-starch interactions and rheological response

Figure 2d shows the general rheological response (Torque, Tq) values measured with the Mixolab at the respective sampling points. All the effects underlying torque-levels during a mixing assay, let alone a mixing + heating assay, are far from clear. It is difficult to experimentally reveal interaction effects between starch and protein in one type of rheological test; hence we used a combination of microscopy and protein extraction to improve our understanding of dough structural changes at the mesoscopic level. A simplified, but often used concept is that of discriminating the effects into two zones: 1) gluten development (C1), overmixing and 2) upon heating, the Tq responses are related to starch swelling/gelatinization only. This simplification should be viewed with some caution. The pattern of Tq vs time-temperature and the de-aggregated / re-aggregated glutenin levels in Fig 2a-d strongly suggests that also proteins must affect the Tq levels beyond C1 (gluten development). For example at sample point #3 we can notice that the Tq response follows: Gruau Rouge > Corde Noire > Ficelle Verte. This indicates that with mild heating (30 – 50°C) beyond C1, torque is still affected by: 1) flour protein content, 2) 1.5% SDS soluble glutenins, especially noted for Ficelle Verte with the lowest percentage of 1.5% SDS solubles at sample point #3 in Figure 2b. At sample point #7=C4 when the dough is processed at high temperatures (80-90°C) it can

420 be noticed that the Tq values for Gruau Rouge = 1.60 Nm and Ficelle Verte = 2.02 Nm.
421 This difference cannot be explained by starch dilution, due to protein content difference
422 alone. Also the amount of 1.5% SDS solubles is similar at this point, but for Gruau
423 Rouge there is a lower recovery of GMP wet weight. A lower swelling in 1.5% SDS
424 indicates that the glutenins are in a highly heat-aggregated state, these heat-aggregated
425 structures may interfere with the consistency of the gelatinized starch phase, hence the
426 lower Tq value observed.
427
428 **Microstructure changes during mixing, heating and cooling**
429 The changes of the microstructure of the main components of the three different wheat
430 flours along mixing-heating and cooling were analysed by different microscopy
431 techniques, which comprised light and fluorescence microscopy, polarized microscopy
432 and scanning electron microscopy (SEM).
433
434 The microscopic images (Figure 3) show the starchy material after staining with lugol.
435 In the wheat flour samples (Figure 3A) two different populations of starch granules
436 were detected, the smaller ones with rounded shape and the bigger granules with
437 lenticular shape. The images obtained during mixing, heating and cooling showed the
438 changes underwent by the starch granules when subjected to mechanical and thermal
439 constraints. The images for the dough mixing (Figure 3B) still showed the two granules
440 population, as well as after the mild heating that occurred in C2 (Figure 3C). When
441 heating proceeded further than 53-55ºC, depending on the flour, where gel formation
442 occurred starch granules showed bigger size due to the swelling phenomenon, which
443 also induced the deformation of the granules (Figure 3D). The remaining granules were
444 surrounding by a more transparent film, which corresponded to the amylose leached out

445 into extragranular space during the starch gelatinization. In C4 (Figure 3E) and C5
446 (Figure 3F) that effect was even more dramatic and the remnants of collapsed granules
447 dispersed in the extragranular polymer matrix were clearly visible, and the initial dark
448 blue colour changed to light pinkish purple colour; suggesting differences in the chain
449 length of the polymers that complexed with iodine, which agree with previous
450 observations of Dillon et al (2011). This technique did not allow differing among the
451 different wheat flour samples.
452
453 Starch granule morphology and birefringence were studied using a polarized light
454 microscope (Figure 4). In Figures 4A, 4B, 4C, it was observed the birefringence in
455 starch granules viewed by polarized microscopy, which indicated the integrity of the
456 starch granules. Two size populations were detected during mixing and mild heating
457 (C2). However, when gelatinization took place, the bigger starch granules lost the
458 birefringence paste, whereas it still was observed in the smaller size population of starch
459 granules. Some birefringence was also detected in C4, but only a few granules of small
460 size, kept that property after heating (Figure 4E).
461
462 The SEM technique allowed to visualize the three dimensional structure of the wheat
463 flour and dough besides the changes induced by mechanical and thermal constraints.
464 Wheat flour appeared as aggregates of protein matrix embedding groups of cellular
465 components, mainly starch granules (Figure 5). In the wheat flours (Figure 5A, 6A,
466 7A,), two distinct populations of starch granule sizes were detected, the larger or A-type
467 granules (lenticular shaped) and the smaller or B-type granules (spherical shaped) on the
468 surface of the A-type granules. Some starch granules appeared distorted as a
469 consequence of milling. Those results agree with previous findings of Rojas et al

(2000). When comparing the different wheat flours, it seems that the starch granules are more disaggregated in flour with the lowest protein content (Ficelle Verte). The other flours showed more compact structure with more cementing material holding the structure, which corresponded to the protein matrix. After mixing (Figure 5B, 6B, 7B), the resulting dough presented a reticular structure where starch granules are embedded in a protein matrix. Numerous holes were observed in that network that derived from the air incorporation during mixing. The starch granules appeared dispersed in the continuous matrix. Again, starch granules were more visible in the sample with lowest protein content (Ficelle Verte, Figure 6B), due to the lower amount of viscoelastic protein material for holding the starch granules. Beyond this stage no structural differences among the different wheat flours were detected. When dough was subjected to heating, protein aggregation followed by denaturation was taking place, however SEM micrographs did not allow to clearly distinguish those changes (Figure 5C, 6C, 7C), nevertheless some smooth areas could be detected, which might be consequence of the gel structure of denatured proteins. At this stage no changes in the starch granules were observed, thus no gelatinization was taking place. In C3, where the starch gelatinization was supposed to occur, changes were readily evident in the dough microstructure. Micrographs (Figure 5D, 6D, 7D) showed swollen and slightly elongated starch granules with distorted structure, they adopted flatten microstructure, where a deep longitudinal groove in the middle could be observed in some granules. At that stage, fragments of proteins were scattered over the starch granules surface, adopting filamentous shapes. No significant differences were observed among the micrographs of doughs from C3 stage and C4 stage (Figure 5E, 6E, 7E). It seems that the additional changes induced when keeping dough at heating affected more the internal structure of the starch granules, but not the external appearance of the granules.

Conversely, after cooling (C5) the microstructure was totally different (Figure 5F, 6F, 7F). Starch granules were completely distorted and only few granules could be envisaged in the dough microstructure. Both A-type and B-type granules were longer and presented a higher dispersion of sizes than in flour and dough. Micrographs showed a combination of smooth zones resulted from the starchy gel, with some cavities linked together by filamentous structures.

The rheological analysis of three different wheat flours by using the Mixolab® device allowed identifying the role of the proteins and the relationship between the protein content and different primary and secondary parameters obtained from the recorded curves. The microstructure analysis using light, polarized and scanning electron microscopy revealed the changes that proteins and starch undergo during mixing, heating and cooling. By polarized and light microscopy it was possible to identify the gelatinization of the starch, whereas the scanning electron microscopy made it possible to observe the three dimensional changes in the wheat dough when subjected to mechanical and thermal constraints. The microstructure techniques did not allow us to draw a firm conclusion on differences in starch structural changes between for example a high vs. lower glutenin wheat flour (GR vs FV). This is plausible, because wheat starch composition of high vs. low protein and glutenin flour can be expected to be similar. Nevertheless, it was possible to observe (Figure 6A, 6B) some differences that are likely to be related to the respective protein content of the flour. It would require a set of wheat flour samples that largely differ in glutenin content to strengthen this finding.

Conclusions

The Mixolab instrument can be used to reproducibly prepare mixing and heat processed dough samples for further study. Industrial dough processing is complex, but we did find valuable information by a systematic microscopy study and determining protein extractability of the dough samples. Qualitatively the starch structural changes, swelling and gelatinization observed by microscopic techniques, shows some parallels with protein (and glutenin) content of the respective flour. Nevertheless, this tentative finding needs further confirmation by studying flour samples with a large difference in glutenin content. The Tq values measured during both mild temperature range (30-50°C) and higher temperatures (70-90°C) of the assay, seem to be affected by both starch and protein structural changes. Unexpectedly, the weakest flour (Ficelle Verte) with the least insoluble glutenin, showed the highest rate of heat-induced (30-50°C) insoluble glutenin recovery rate. Our findings indicate that effective protein dispersing and dough consistency are important in determining glutenin aggregation rate during the Mixolab assay. This demonstrated that studying on the meso- and macro level has advantages over studies attempting to find answers on macro-rheological phenomena at the molecular level of SH groups. On basis of protein mass conservation in a dough system we must consider that albumins and globulins have ‘co-aggregated’ with SDS insoluble glutenin.

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FIGURE CAPTIONS

Figure 1. Schematic plot of a generic Mixolab® curve and points of sampling for protein extractions and microscopy analysis.

Figure 2.

2a) Relative percentages of SDS insoluble GMP-gel wet-weight from Mixolab® doughs at various mixing stages for flour samples Gruau Rouge, Corde Noir and Ficelle Verte, taking flour GMP-gel wet weight as 100%.

2b) Relative percentages of SDS soluble protein (SDSS) from Mixolab® doughs at various mixing stages for flour samples Gruau Rouge, Corde Noir and Ficelle Verte, taking flour SDSS as 100%.

2c) Relative percentages of water-soluble protein (WS) from Mixolab® doughs at various mixing stages for flour samples Gruau Rouge, Corde Noir and Ficelle Verte, taking flour WS as 100%.\

2d) A plot of the general rheological mixing pattern (Torque, Tq) vs sampling points for the protein extraction study – split in a low Tq section (left) and higer Tq section (right).

Figure 3. Light micrographs of wheat flour (A) and wheat dough (B-F) from Gruau Rouge. Wheat dough obtained from the Mixolab® at stage C1 (B), C2 (C), C3 (D), C4 (E), C5 (F). Starch was stained with lugol. Micrographs magnification 40x.

Figure 4. Polarized micrographs of wheat flour (A) and wheat dough (B-F) from Gruau Rouge. Wheat dough obtained from the Mixolab® at stage C1 (B), C2 (C), C3 (D), C4 (E). Micrographs magnification 40x.

Figure 5. Scanning electron micrographs of wheat flour (A) and wheat dough (B-F) from Gruau Rouge. Wheat dough obtained from the Mixolab® at stage C1 (B), C2 (C), C3 (D), C4 (E).

Figure 6. Scanning electron micrographs of wheat flour (A) and wheat dough (B-F) from Ficelle Verte. Wheat dough obtained from the Mixolab® at stage C1 (B), C2 (C), C3 (D), C4 (E).

Figure 7. Scanning electron micrographs of wheat flour (A) and wheat dough (B-F) from Corde Noire. Wheat dough obtained from the Mixolab® at stage C1 (B), C2 (C), C3 (D), C4 (E).

Table 1. Scheme of sampling performed for protein extractability and microstructure studies (SEM and LM). Dough samples were taken after reaching the Mixolab parameter point and further used for protein or microstructure analysis.

Sample No.	Mixolab Parameter	Description	Protein Extractions	SEM and LM
1	C1	Dough peak resistance at 30°C	+	+
2	C1->C2	Onset of dough weakening	+	-
3	C1->C2	Further thermo-mechanical weakening	+	-
4	C2	Dough weakening minimum	+	+
5	C2->C3	Dough at intermediate stages of thermal pasting	+	-
6	C3	Dough at the peak of thermal pasting	+	+
7	C4	Dough viscosity at peak dough temperature	+	+
8	C5	Dough viscosity increase at cooling	+	+

665 **Table 2.** Wheat flour characteristics and Mixolab® parameters of three different

666 commercial flours.

	Gruau Rouge		Ficelle Verte		Corde Noire	
	Mean	SD	Mean	SD	Mean	SD
Absorption (%)	55.8	0.1	53.0	0.1	54.2	0.1
Time to C1, min	1.6	0.1	1.0	0.1	1.2	0.1
C1, Nm	1.13	0.04	1.09	0.03	1.12	0.02
Stability, min	10.8	0.3	6.3	0.9	6.9	0.7
C2, Nm	0.55	0.02	0.44	0.01	0.41	0.01
Time to C2, min	17.4	0.0	17.0	0.1	17.5	0.0
Temperature at C2, ºC	54.8	0.8	53.4	0.7	55.0	0.7
Initial pasting temperature, ºC	60.3	0.5	60.7	0.4	62.2	0.6
C3, Nm	1.96	0.03	2.06	0.02	1.99	0.03
Time to C3, min	25.0	0.1	26.0	0.1	26.1	0.1
C4, Nm	1.61	0.03	2.02	0.04	1.93	0.10
Time to C4, min	31.4	0.1	30.9	0.1	31.9	0.1
C5, Nm	3.04	0.02	3.01	0.01	2.66	0.04
Time to C5, min	45.0	0.0	45.0	0.0	45.0	0.0
Protein weakening range, C2-C1, Nm	-0.59	0.03	-0.66	0.02	-0.71	0.02
Starch gelatinization range, C3-C2, Nm	1.42	0.02	1.62	0.01	1.58	0.02
Cooking stability range, C4-C3, Nm	-0.34	0.06	-0.04	0.04	-0.05	0.07
Pasting temperature range, ºC	25.6	0.9	28.7	1.8	27.8	2.8
Gelling, C5-C4, Nm	1.42	0.06	1.02	0.01	0.81	0.01
alpha, Nm/min	-0.091	0.002	-0.068	0.004	-0.078	0.004
beta, Nm/min	0.518	0.034	0.453	0.033	0.510	0.039
gamma, Nm/min	-0.204	0.010	-0.015	0.004	-0.017	0.005
delta, Nm/min	0.115	0.017	0.079	0.004	0.076	0.002
Tenacity (P), mm	76	5	57	3	60	4
Extensibility (L), mm	165	8	157	6	155	4
P/L	0.46	0.03	0.36	0.02	0.39	0.03
Deformation energy (W), $x10^{-4}$ J	358	10	212	9	214	8
Damage starch, %	21.50	0.02	22.80	0.42	23.50	0.32
Ash, %	1.13	0.00	0.95	0.01	1.00	0.01
Protein, %	14.90	0.02	9.89	0.08	10.96	0.00
Carbohydrates, %	69.27	0.08	75.14	0.18	73.23	0.12

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668 Mean values within rows were significantly different at $P<0.05$

669 Figure 1.

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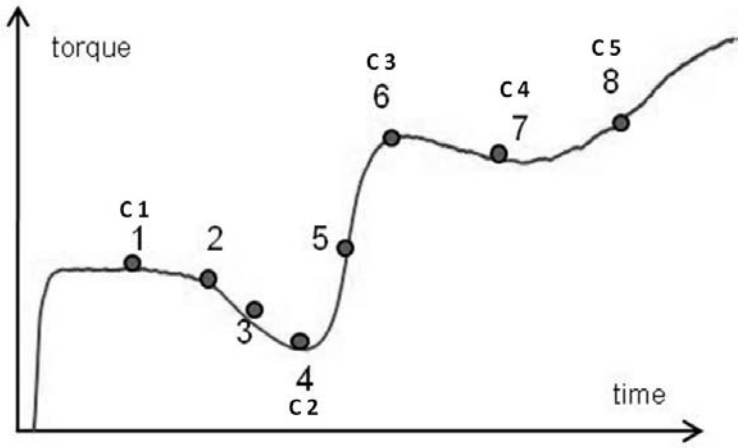

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675 Figure 2a-d

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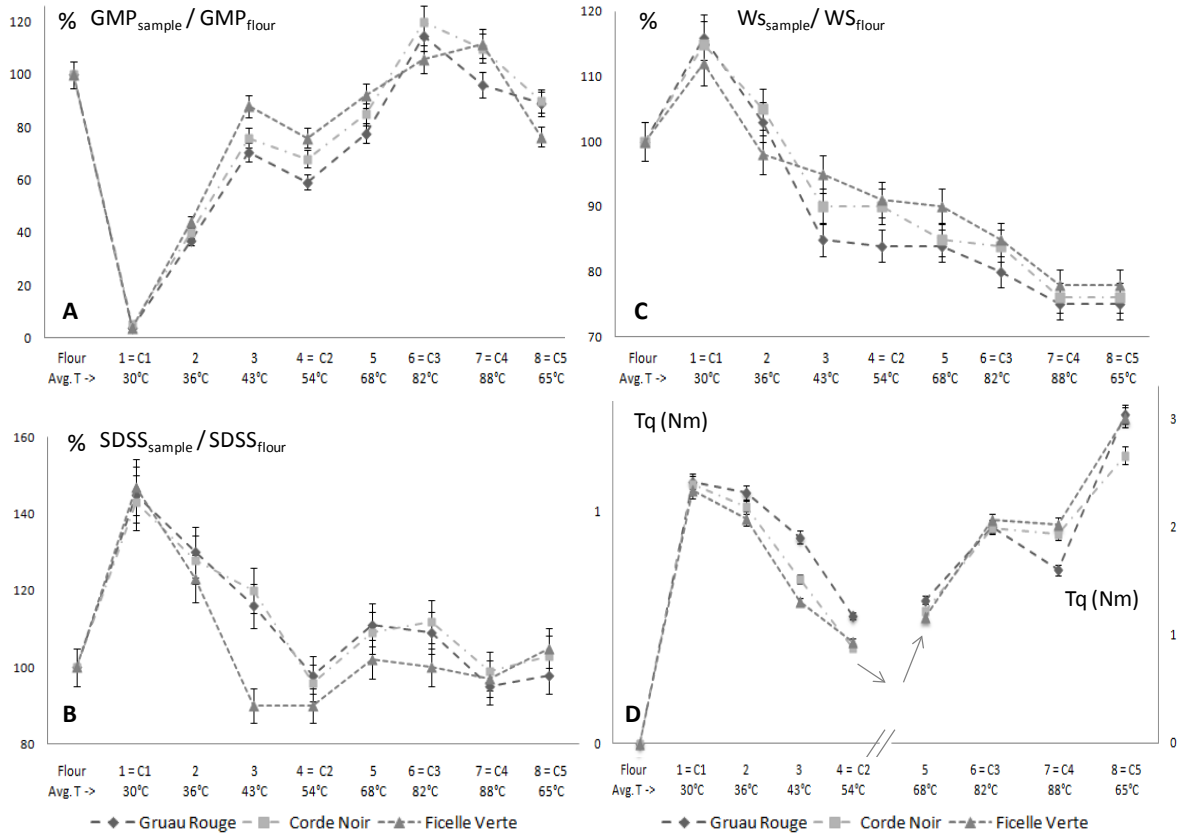

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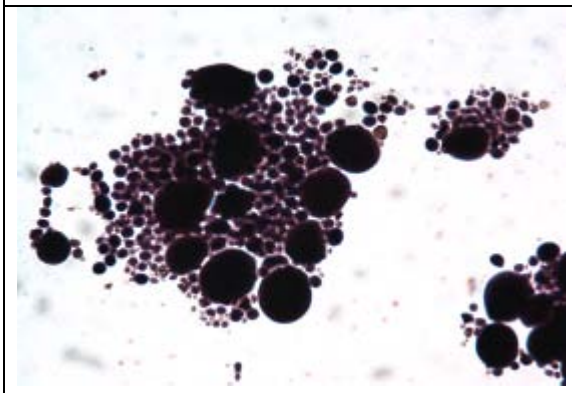	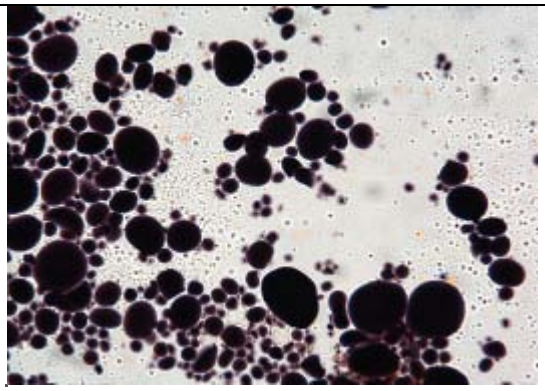
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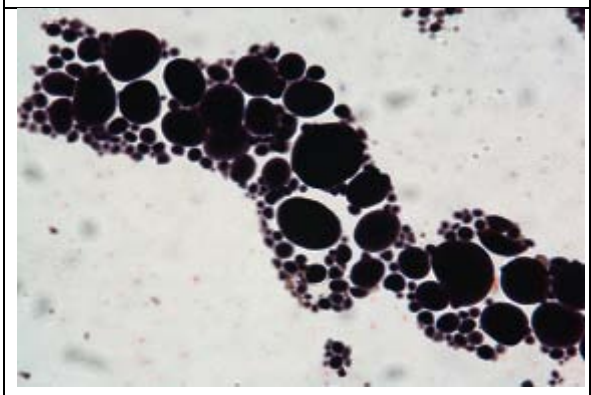	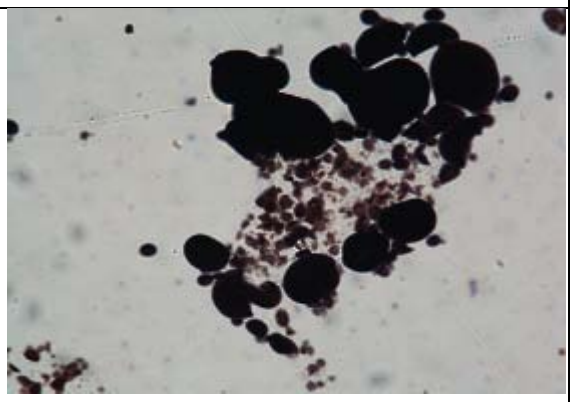
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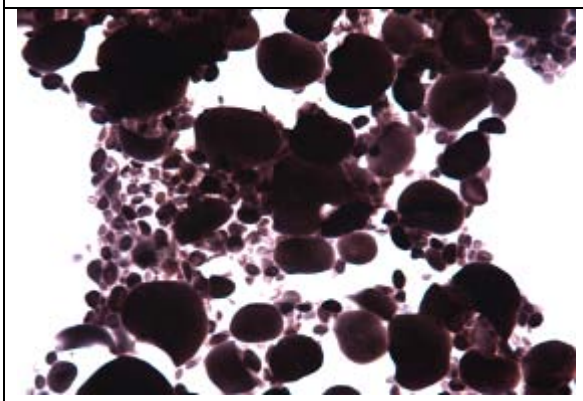	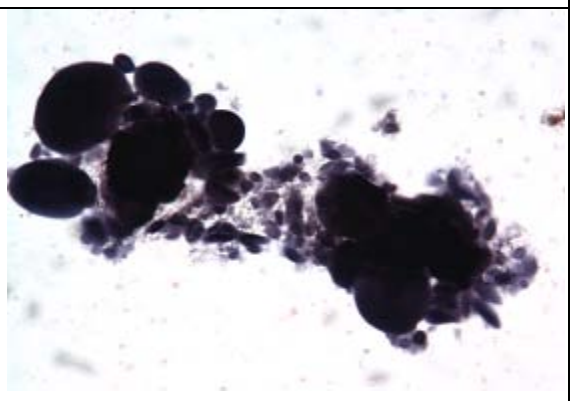

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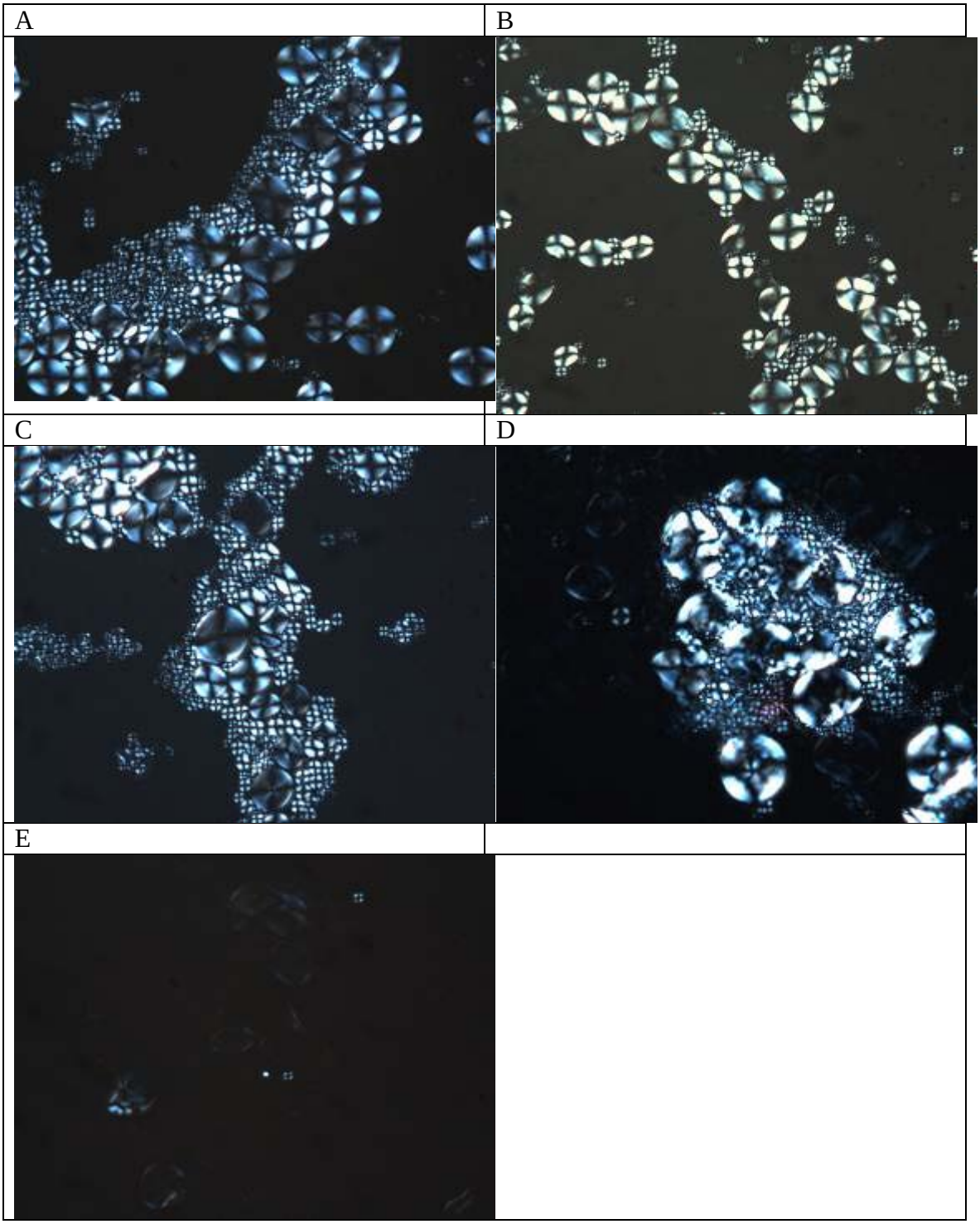

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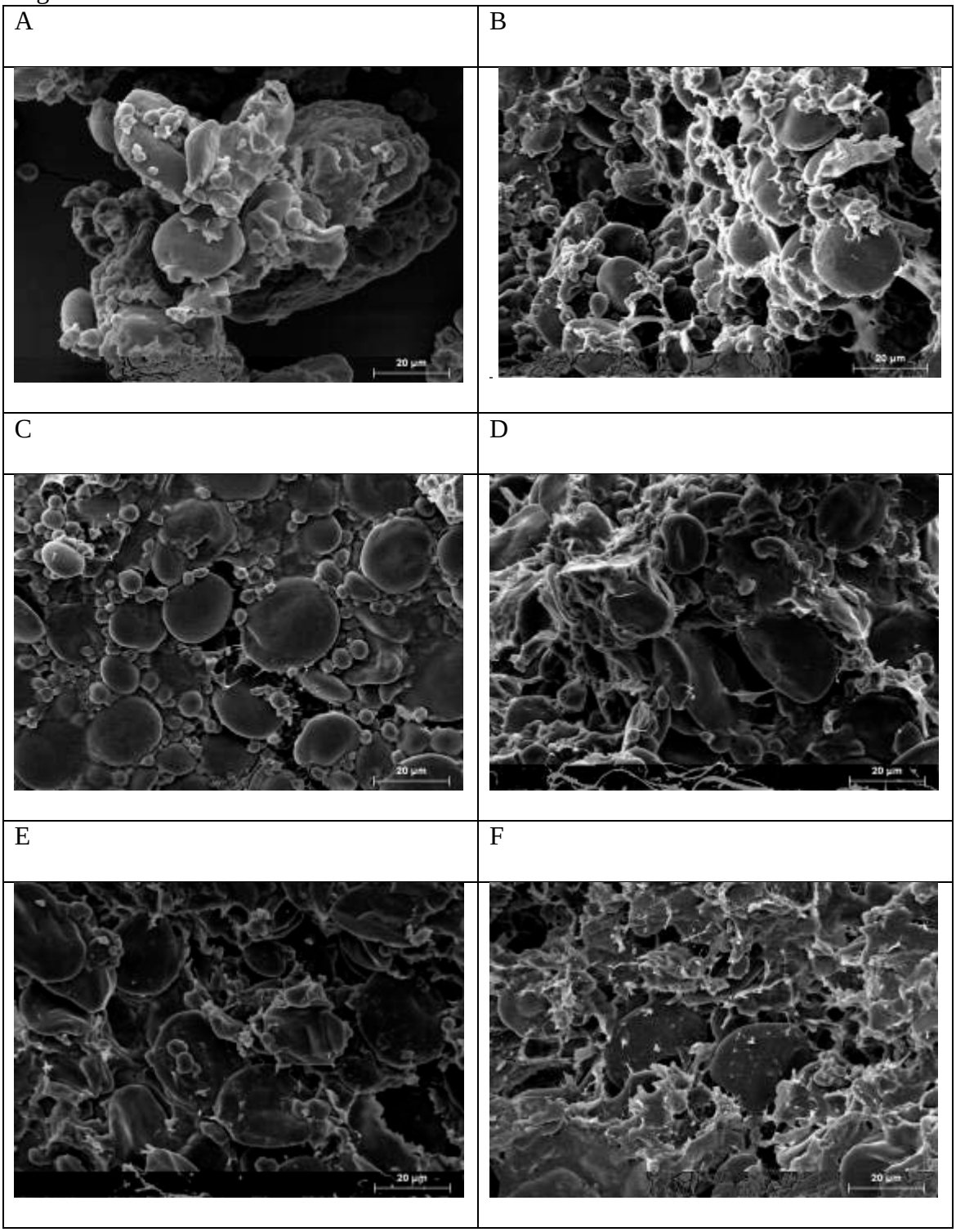

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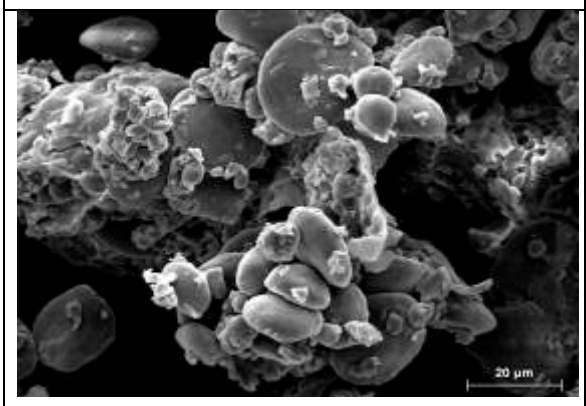	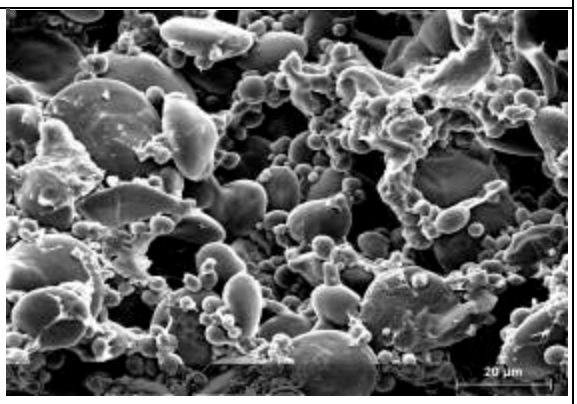
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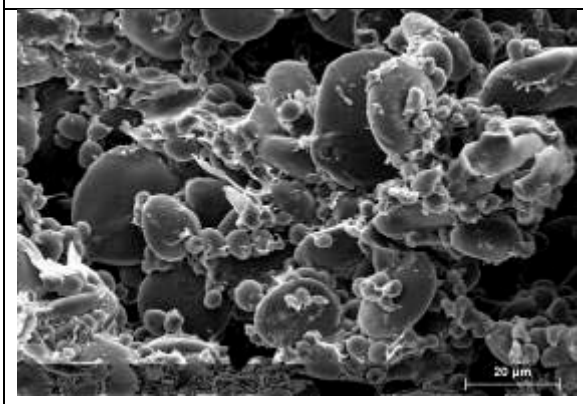	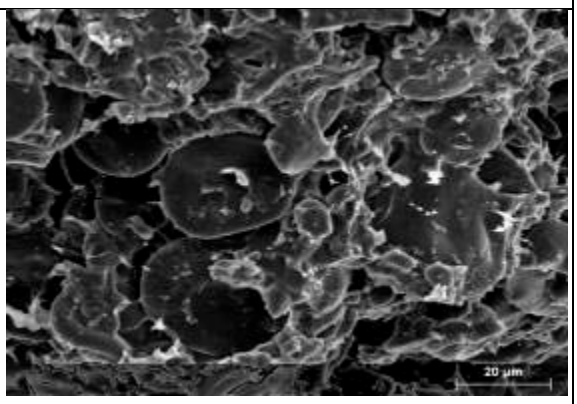
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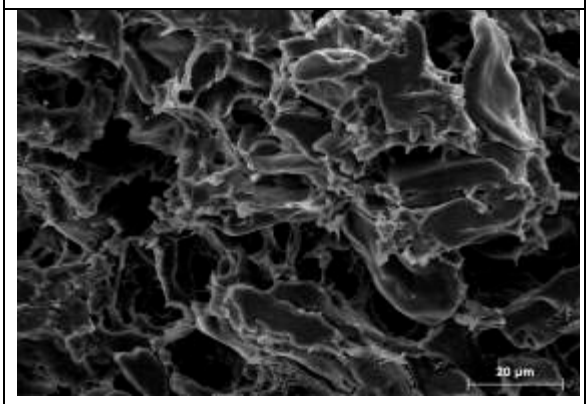	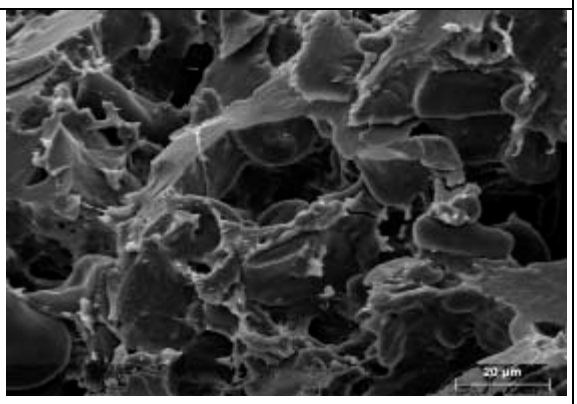

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693 Figure 7.

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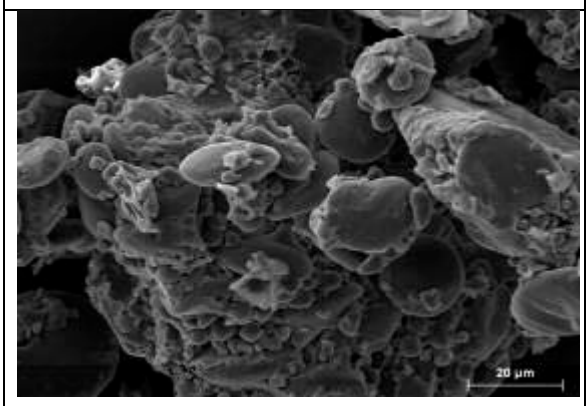	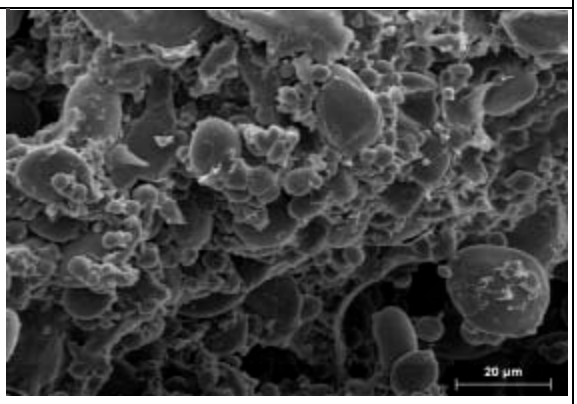
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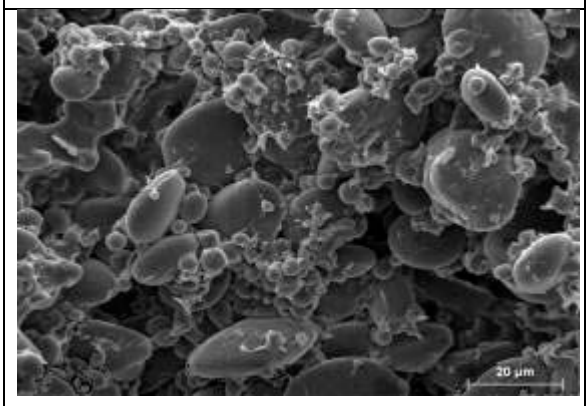	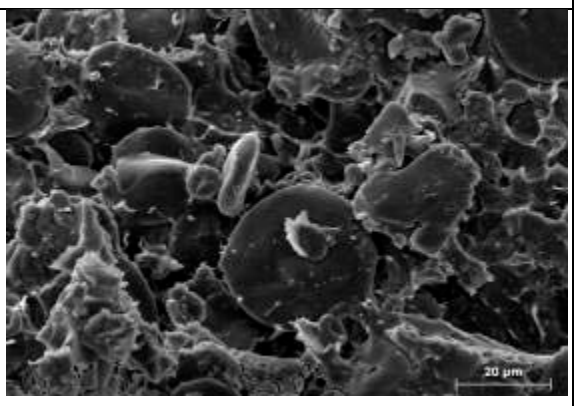
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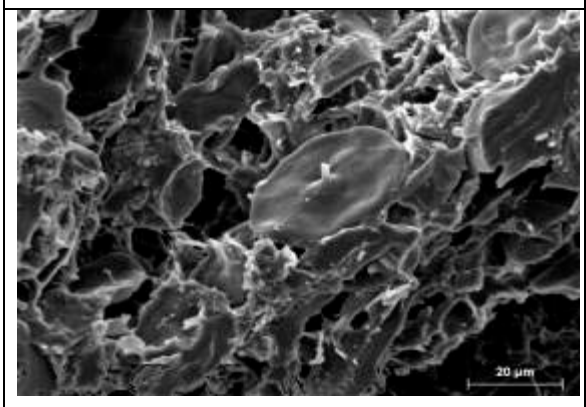	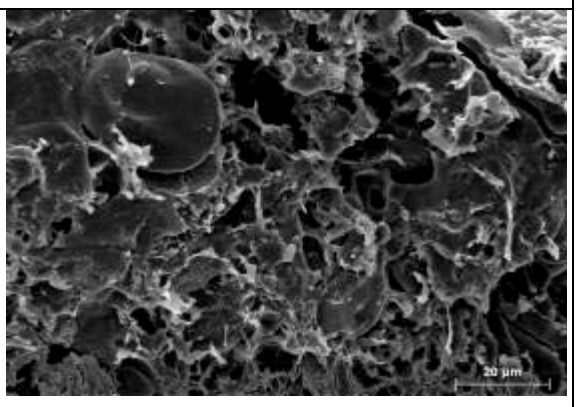

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