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Jean-Baptiste Boitte, Murielle Hayert, Camille Michon. Observation of wheat flour doughs under mechanical treatment using confocal microscopy and classification of their microstructures. *Journal of Cereal Science*, 2013, 58 (3), pp.365-371. 10.1016/j.jcs.2013.07.009 . hal-01173802

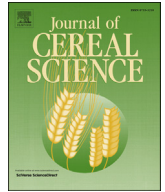
HAL Id: hal-01173802

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Observation of wheat flour doughs under mechanical treatment using confocal microscopy and classification of their microstructures



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ARTICLE INFO

Article history:

Received 11 April 2013

Received in revised form

9 July 2013

Accepted 23 July 2013

Keywords:

Cereal

Rheo-optic

Gluten network

Image texture analysis

Mathematical morphology

ABSTRACT

RheOptiCAD[®] is a cell allowing observation of microstructures under thermo-mechanical treatments. Using the device fitted on a confocal microscope, wheat flour doughs containing 3 levels of water were studied. Dough were placed between 2 parallel plates and submitted to motion of the bottom one. Temperature was controlled and fixed at 20 °C. Methodology and experimental parameters, but also balance between optical and mechanical ones are described and justified to set a dynamic observation in real time. Rhodamine B labeled gluten network was observed under dynamic mechanical treatment, i.e. a 0.4 mm-0.3 Hz oscillation applied during 30 s. The evolution of the gluten network microstructure along time of deformation and depending on the recipe was studied. Image sequences are presented to visualize the qualitative differences in the network. Differences in microstructures were observed. A grey scale mathematical morphology is proposed to qualify and quantify these differences. Images can be separated according to the level of orientation of the network and to the local protein concentration in the gluten fibers.

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1. Introduction

Wheat flour dough results from the mixing of wheat flour and water. Dough is processed according to 3 main steps: kneading, fermentation and baking. Kneading is the principal step consisting of mechanic action which ensure homogenization, airing and structuration of the dough. It leads to the formation of the gluten network, created by several inter- and intramolecular disulphide bounds between proteins of the flour (glutenins, gliadins), respectively. Stability of the gluten network is also due to non covalent bounds as hydrogen or hydrophobic bounds and entanglement between chains Bloksma (1990). Resulting wheat flour dough is macroscopically homogeneous and microscopically made of a dispersion of air bubbles themselves dispersed in a network made of starch granules confined in a protein matrix. Wheat flour dough has viscoelastic properties which are mainly dependent on the

composition in glutenin and gliadin but also in water. The major constituent of wheat flour is starch, but it is rather the protein fraction that is responsible of the formation of dough. Starch granules play a key role during the baking stage (gelatinization process occurring when the temperature is raised) but not so much during the mixing stage. One could also consider interactions between pentosan and gluten, which lead to a competition in the hydration process, and thus an effect on the rheological properties of the dough.

Rheological properties are notably driven by the water content. High content is favoring protein mobility and as a consequence decreasing the apparent viscosity of the dough, at least at small deformations. To mimic the deformation (bi-axial extension, ϵ_b) of a layer of dough sheared between bubbles during fermentation, lubricating squeezing flow (LSF) is a powerful technique. LSF could scan a wide range of deformation and Lassoued (2005) studied the apparent viscosity of dough according to the deformation level for different water contents. For a small ϵ_b , apparent viscosity decreased with increase of water content. However, they also observed that dough enriched in water (60%) gets a large increase of its apparent viscosity from $\epsilon_b = 0.3$, which corresponds to a strain hardening behavior that is not observed for lower water contents. A

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correlation with microstructures was attempted by performing confocal microscopy, but in separate experiments and without deformation of the dough while observing.

Lassoued (2005) showed that microstructure of the dough was depending on the water content. Confocal laser scanning microscopy (CLSM) is widely used to observe microstructures of multiphasic systems such as food. Numerous papers were published about observation of complex and heterogeneous systems such as food products using confocal microscopy as well as labeling techniques (for example in, Heertje, 1993; Tromp, 2001; van de Velde et al., 2003; Blonk and van Aalst, 1993; Durrenberger et al., 2001). Wheat flour or bread dough was extensively studied by CLSM. Covalent labeling of the gluten network was performed by Li et al. (2004) using Rhodamine B and by Dubreil et al. (2002) using RhodolGreen. Nevertheless, sample preparation often present some disadvantages inducing artifacts in the microstructure. Squeezing, stretching or dehydrating while waiting for labeling diffusion and/or observation can happen and lead to an induced orientation of the gluten network or to a non-representative information. In this way, controlling the thermo-mechanical environment of a sample during its observation is of interest. Coupling rheology and microscopy in order to illustrate modifications of the structure of a complex system such as bread dough is highly interesting to understand involved mechanism during processes and effect of ingredients on the microstructure. In most of the studies, rheological characterizations were carried out apart from structures observations. Autio and Laurikainen (1997) did a review about dough structures and the correlation with their compositions (lipids, bran, enzymes) or processes (mixing, fermentation, baking). They conclude on the need of dynamic studies at different scales but also on the quantification on structural parameters through image analysis to obtain data on the structural components of dough or bread. Water content effect on rheology and microstructure was studied by Letang et al. (1999) taken into account the time of development of the dough. Using ESEM pictures, a molecular model of gluten network was proposed. More recently, Upadhyay et al. (2012) have studied the effect of water, yeast and hydrocolloids (alginate and xanthan) on the viscoelastic properties (dynamic rheology) and air bubbles distribution (CLSM + images analysis). According to their work, increasing water content decreased the modulus but increased size bubbles whereas yeast has an opposite effect. Lee et al. (2001) used a CSLM to study relationships between extensional and flow properties of dough and corresponding microstructures at different level of development of the dough. Since 2004, Peighambardoust, collaborating with different teams, has studied relationships between rheological properties and microstructures of bread dough. Using either a cone-plate home-made device Peighambardoust et al. (2004) or a cone–cone geometry Peighambardoust and van der Goot (2010), formation of dough at different states including migration of gluten and shear effect on the network were studied and observed using CLSM Peighambardoust et al. (2006); Peressini et al. (2008). In the latter works, an elongation of the gluten network was observed and interpreted to be shear rate dependent. Measuring the torque during shearing showed sudden drop in torque values, identified as local breaks in the gluten network.

Objectives of the present work were to observe and characterize the evolution of the gluten network of the wheat flour dough under a plane–plane shear up to large deformation and to study the effect of the water content. A novel rheo-optical device coupling translation shear to confocal microscopy developed by our team Boitte et al., 2013 was used. Dynamic oscillations were imposed to wheat flour doughs. Images of the microstructure were simultaneously recorded. Qualitative description was performed followed

by a quantitative approach using mathematical morphology as a texture images analysis.

2. Materials and methods

2.1. Bread dough samples

Bread making wheat flour was used (Corde Noire, Soufflet, France). The protein content is was $11 \pm 1\%$. Three wheat flour doughs with various amount of deionized water (Merck Millipore, Germany) were prepared. Water content were 50% (dough W–), 55% (dough Std) or 60% (dough W+), in % in flour basis. 2% of salt, provided as NaCl (Sigma–Aldrich, France), was diluted in water before adding the flour.

2.2. Gluten fluorescent labeling

Prior to doughs preparation, gluten was labeled by Rhodamine B (Sigma–Aldrich, UK) using protocol described by Li et al. (2004). Starch granules were eliminated under water from a standard dough (33 g of water and 100 g of wheat flour) leading to gluten network. Gluten was then freeze-dried and 1 g was placed for 7 h in a 0.01% w/v Rhodamine B solution under gentle stirring and away from light. Labeled protein solution was then dialyzed in water at 4 °C during 72 h and finally freeze-dried again to obtain a pink powder. In these conditions, no free Rhodamine B is present, preventing from interaction with starch granules when mixing with flour. For each dough recipe, 25 mg of labeled gluten was added to 10 g of flour. Rhodamine B was excited by a He/Ne laser line (543 nm) and emitted from 560 nm with a maximum of emission between 575 and 600 nm, range where photomultiplier was settle.

2.3. Dough preparation

Using a Mixograph (National MGF Co., USA), 10 g of wheat flour and 25 mg of labeled gluten were pre-mixed during 30 s at 88 rpm. Water containing dissolved salt was added and mixed during 4 min 30 s at 88 rpm. The mixing process was reproduced from Lassoued (2005). As no tools could really give information on the optimal mixing time, we have used our expertise of bread making and determined the needed mixing time by sensory feeling of the dough. For a fixed mixing time, a 55% water content dough is elastic, extensible, lowly sticky. When adding water, we obtained humid and sticky dough whereas decreasing to 50% lead to coarse, granular, non-cohesive dough.

Doughs were then kept at 4 °C in a fridge for at least 1 h Davidou et al. (2008) before any rheo-optical measurements. This ensures dough to relax from all stresses caused by the mixing process.

2.4. Observation under shear

Using a home-made designed device, called RheOptiCAD[®] (Boitte et al., 2013, Fig. 1), observations under shear were performed at controlled temperature. The designed shear cell was placed on top of an inverted confocal microscope Leica TC SP2 (Leica, Germany). The samples were placed between 2 parallel plates, the bottom one allowing observation through a hole. A small piece of dough was cut with care using sharp scissors and positioned in the center of the bottom plate. The top plate moved down at a constant and low speed (2 mm.s) to prevent from an excessive compression of the sample. Gap width was set at 500 μm and resting time before measurement was 30 min. Positioning a sample of dough within the gap engendered squeezing stresses on it and bending of the bottom microscopy slide. Thus, resting time was

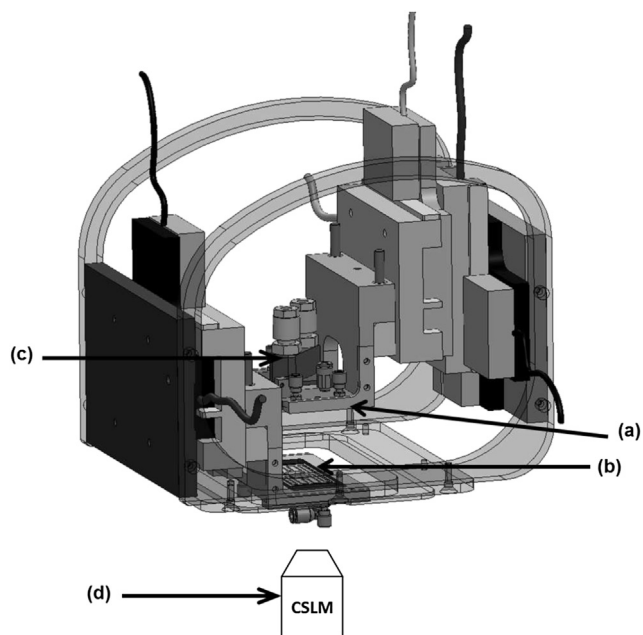


Fig. 1. 3D-scheme of the thermo-mechanical cell placed on top of an inverted CSLM – (a) Top plate; (b) Bottom plate; (c) Peltier + water cooling system; (d) Objective of the CSLM.

determined to be sufficient enough to recover from squeezing and relax from bending. Bottom plate oscillations were performed as mechanical treatment ($f = 0.3$ Hz and $A_{\max} = 0.4$ mm during 30 s). This oscillatory deformation ($\gamma_0 = 0.8$) is relevant compared to the level of deformation observed during fermentation process [Blokma and Bushuk \(1988\)](#) and to the elongation–compression cycles happened during mixing.

A water immersion objective $\times 40$ (0.8 NA) coupled to a 543 nm He/Ne laser line were used to observe flour dough, especially the labeled gluten network that was uniformly spread inside the protein network. Optical parameters were set in balance with mechanical ones, in order to obtain a sized enough image to visualize the gluten network but not too large to be able to record enough images per second to follow the evolution of the network. Due to these considerations, images, sized 512×256 pixels, were acquired every 250 ms, equivalent to 13 images per period of oscillation, *i.e.* 130 images for a complete mechanical treatment applied on a dough sample.

After resting doughs for 30 min in order to relax from stresses issued from the sampling and the positioning between the 2 plates of the shear cell, dough microstructure was observed before any further deformation. High consistency and dough opacity prevent to visualize in a deep focal plane. However, to avoid artifacts due to the contact between the dough and the microscopy glass, observation was performed up to 5–10 μm from the bottom plate.

2.5. Images and data analysis

Inspired from previous papers of [Devaux et al. \(1997\)](#), [Devaux et al. \(2008\)](#), images texture analysis was performed using grey level mathematical morphology. This technique was also applied on bread crumb [Lassoued \(2005\)](#); [Lassoued et al. \(2008\)](#); [Rouille et al. \(2005\)](#).

Red Green Blue (RGB) images resulting from the acquisition along time of bread dough microstructure were converted in grey level. Then, using a program developed by [Devaux et al. \(1997\)](#),

[Devaux et al., 2008](#) running on MATLAB (The MATHWORKS, USA), all images were analyzed with grey level mathematical morphology. Images were submitted to 30 successive steps of erosion and dilation probing with a linear structuring element of 1 pixel radius and an angle of 135° or 45° depending on the orientation of the image. An erosion decreases the total grey level of the image leading to a complete black image whereas a dilation will increase this grey level to a complete white image after 30 steps. Erosion causes a size reduction of bright object when dilation is the dual effect by reducing size of dark object. In the present case, erosion will look at the morphology of the gluten network, corresponding to the bright pixels. In the contrary, focusing on dilation will give information about the holes within the network (dark pixels). The variation of the grey level sum between 2 steps, g_i is calculated following Eq. (1).

$$g_i = \frac{V_i - V_{i+1}}{V_0 - V_f} \quad (1)$$

where V_i and V_{i+1} are respectively the sum of grey level at step i and $i+1$, V_0 and V_f are the initial and final sum of grey level of the image. Plotting g_i as a function of dilatation and erosion steps, *i.e.* as a function of the size of the structuring element, leads to a granulometric curve. Doing these operations for the doughs and all images along shearing time gave a set of 390 granulometric curves (3 doughs \times 13 images). All data were submitted to a principal component analysis (XLStat, Addinsoft, France) to get a relevant comparison of the morphology of the microstructures. Using the scores obtained for all images on the 2 first components, maps were obtained and interpreted.

3. Results and discussion

3.1. Initial microstructures depending on the recipe

[Fig. 2](#) shows images of the doughs containing different water concentration before the mechanical treatment. Gluten network is in white whereas dark zones represent starch granules and air bubbles.

Standard dough (Std), containing 55% of water (in flour basis), has a discontinuous gluten network where dark zones are regularly distributed and spread in the white network all over the image. Amount of labeled gluten mixed with the flour was the same for all

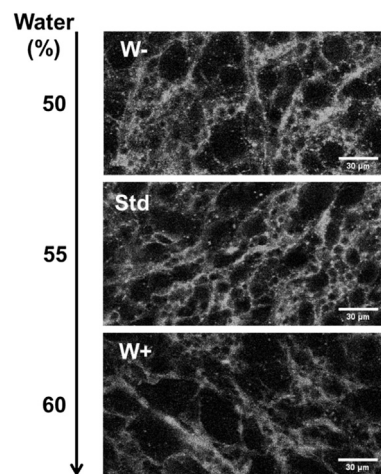


Fig. 2. Microstructures of wheat flour doughs before mechanical treatment depending on their water content. White: gluten network labeled by Rhodamine B. Black: starch granules and air bubbles. Objective: $\times 40$ (water). Images = $220 \mu\text{m} \times 110 \mu\text{m}$.

recipes and the laser intensity were also set at the same value. As a consequence, color intensity of the network of standard dough serves as reference, comparison between color intensity can be made and indicated a certain level of protein concentration. By decreasing the water content (W− dough), the white intensity zones seem sharper forming sort of strands, as if the protein of the gluten network were more concentrated in broad strands. By increasing water (W+ dough), the network is interrupted by bigger dark zones between white strands, giving an impression of heterogeneity within the dough. The effects of water content on microstructure are in good accordance with the microstructures showed in Bloksma (1990); Lassoued (2005).

3.2. Observation under mechanical treatment

Fig. 3 shows images sequences of gluten network evolution depending on the water content of the dough, for 2 different half-period of oscillation. The top sequences correspond to the beginning of the deformation (8 images between 1 and 2.75 s of the oscillation) and bottom ones to the end of the series of 9 oscillations (8 images between 28 and 29.75 s of the oscillation).

After a lag time of 1 s due to the starting motion of the bottom plate, images Std_1 and Std_1.25 correspond to the state of the gluten network for maximum deformation ($\gamma = 0.8$) and zero shear rate. Std_2.5 and Std_2.75 are equivalent to Std_1 and Std_1.25 in terms of displacement of the bottom plate, gluten network being non-oriented. Then, considering time between 1.25 and 2.25 s, bottom plate moved in the opposite direction and images Std_1.5 to Std_2.25 show the orientation of the network during the motion. Same behavior is observed for W− and W+ doughs with an

elongation of the gluten network between $t = 1.25$ and $t = 2.25$, i.e. when shear rate is the highest. Comparing Std_1 with W+_1, a gluten network a bit more oriented is already observed for the latter one. This observation is mainly due to the squeezing during setting the gap width. W+ had not completely recovered.

After 30 s of oscillations, images obtained during the last period are compared to the ones of the first period. Qualitatively, gluten network seems to have the opposite behavior, with orientation when deformation is high but shear rate is low. Gluten network is less oriented or elongated between $t = 28.5$ s and $t = 28.75$ s than at $t = 28$ s and $t = 29.5$ s. Otherwise, looking to Std dough, one can observe the strong orientation of the network in Std_29.5 and Std_29.75. Compared to the network between 1 and 2.75 s, the microstructure has changed during this 30 s of oscillation, leading to oriented structures for a same deformation level. The time of oscillation, which could be compared to a wear test, generated modification within the microstructure and lead to a different behavior of the gluten network. Moreover, several differences can be observed depending on the water content. Orientation of W+ gluten network is more pronounced than W− network. Water has an effect on the elongational properties of the dough. For example, comparing W+_1 and W+_29, surface of dark zones increased with the time of oscillation. Mechanical treatment of 30 s on W+ dough should have modified its microstructure by breaks or rearrangements leading to the latter observation.

Qualitative description showed differences in the gluten network behavior depending on the composition of the dough and on the deformation level and shear rate. During mechanical treatment, depending on deformation level and duration, microstructural changes can happen: protein rearrangement, local ruptures

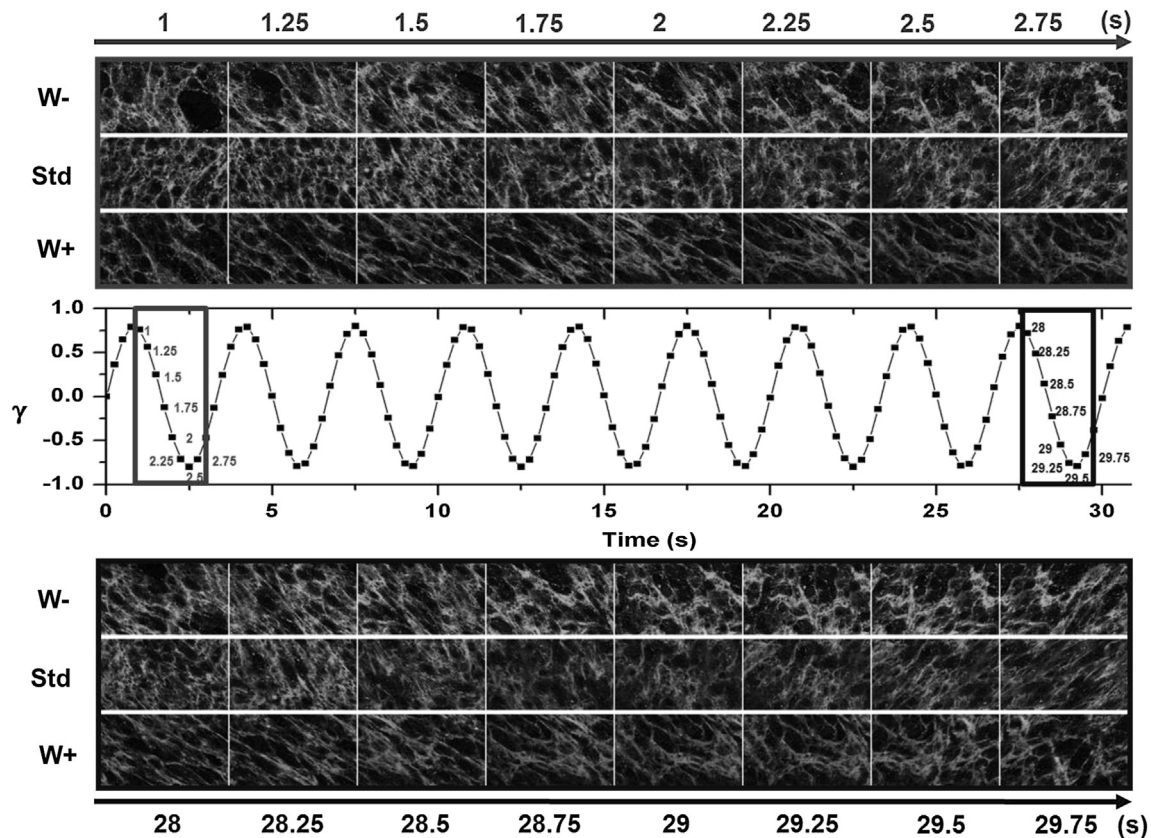


Fig. 3. Image sequences of the microstructures of wheat dough during mechanical treatment depending on the water content for 2 different time period. Top: images between 1 and 2.75 s. Bottom: images between 28 and 29.75 s. White: gluten network labeled by Rhodamine B. Objective: $\times 40$ (water). Images = $220 \mu\text{m} \times 110 \mu\text{m}$. Thereafter, images are named as: X_y with X = Std, W+ or W− and y = time in second. For example, Std_1.75, correspond to the image of standard recipe at time 1.75 s.

of gluten strands, air bubbles and/or starch granules motions, local phase separation ... These changes are responsible for the observed behavior of the gluten network and are correlated to its morphology (thickness of the protein strands, orientation, area of dark/bright zones, local protein concentration). However, no information on the morphology of this network is given by the qualitative analysis. Moving to a quantitative approach may help to clarify these differences.

3.3. To a quantitative analysis using grey scale mathematical morphology

Already used on tomato cells Devaux et al. (2008) or cream cheese Fenoul et al. (2008), grey scale mathematical morphology has never been performed of gluten network behavior. Nevertheless, this image texture analysis has already been applied to bread crumb Lassoued (2005), Lassoued et al. (2008); Rouille et al. (2005) in order to link the effect of water and/or saccharose content and process parameters to the final texture of the crumb. In this case, crumbs were differentiating according to the distribution and the size of air bubbles.

3.3.1. General PCA map and interpretation of PCs

Differences described above were focused on gluten network corresponding to the bright pixels on the images. Thus, only data issued from successive erosion steps, that give the rate of bright object disappearance, were taken into consideration. Images from Fig. 3 were all analyzed and successive erosion data were included in a principal component analysis (PCA) to generate a map of differences of the images (Fig. 4). The 2 first principal components represented 98.6% of the total variance. Principal component 1 (PC1) gives 77.5% of the information whereas PC2 gives 21.1%. Each point corresponds to an image. Data are well spread over the PCA map.

In order to interpret differences in positioning image, all of them were observed. Then, images with extreme position on each axes are shown (Fig. 4). Considering the horizontal axis, one can compare images Std_1 and Std_28.25 with image W+_1. There are differences in the contrast of these images. Images from the right side of the map have a better white contrast. There is a more noticeable gluten network than in images from the left side, which present a more diffuse and less contrasted network. Intensity of the bright pixels in the image placed on the right side verify also this observation. As far as the white color is representing the gluten, labeled by RhoB, interpretation of PC1 as the local protein concentration in the gluten's strands is proposed. Considering now vertical axis, and comparing images Std_1, W-_1 and W+_28.75, a ranking was sort out, from the less oriented gluten network (Std_1) to the more oriented one (W+_28.75). Therefore, PC2 may be interpreted as a level of orientation of the gluten network.

Following this interpretation, effect of deformation level and time of deformation on the microstructure of the 3 different doughs can be analyzed. From Fig. 4, some data were extracted to simplify the reading of it, leading to Fig. 5. On the latter were represented images for, (i) 2 different levels of deformation ($\gamma = 0.8$ and 0.2) at the beginning of the deformation ($t = 1-2.5$ s, filled symbols); (ii) the same deformation ($\gamma = 0.8$) at 2 different times of deformation ($t = 1$ s and $t = 28$ s).

It is interesting to notice that no clear and obvious trend is observed on PCA map (Figs. 4 and 5) according to the water content only. PCA is a multi-dimensional statistical method which represents data (images here) taking into account several parameters such as: time of deformation, protein concentration in the gluten network, orientation of the network and water content.

3.3.2. Effect of the deformation level

Considering images from the first half-period, the effect of the deformation level was studied. For $t = 1$ s corresponding to $\gamma = 0.8$ and for $t = 1.75$ s corresponding to $\gamma = 0.2$, the gluten network

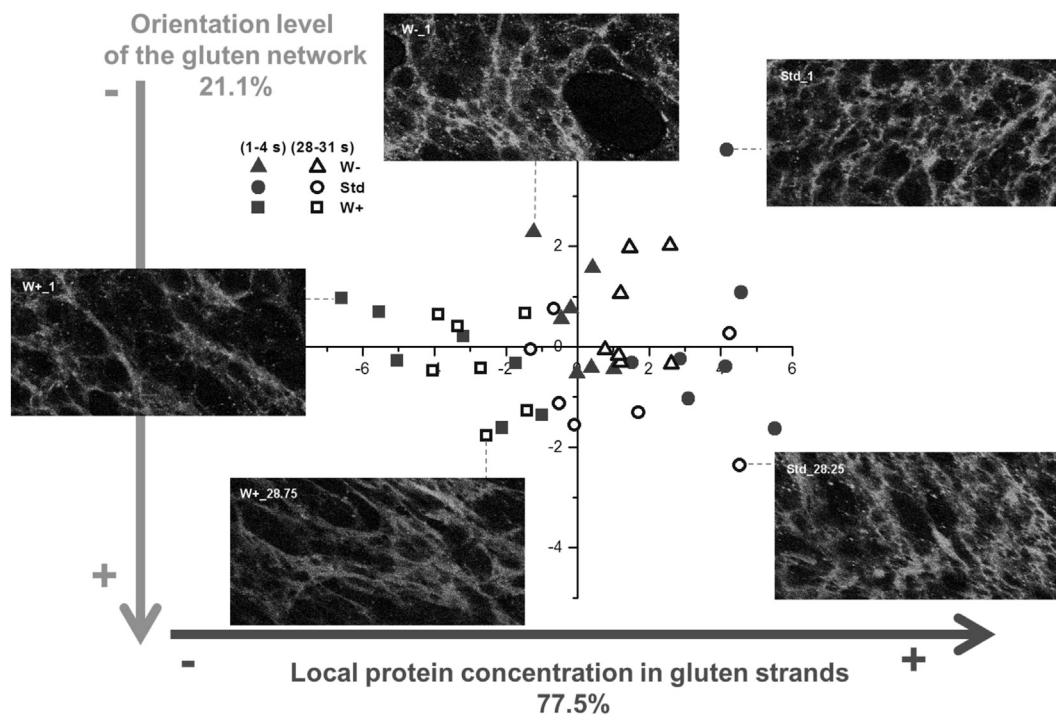


Fig. 4. General PCA map representing images between $t = 1$ and $t = 2.5$ s (filled symbols) and between $t = 28$ and $t = 29.5$ s (unfilled symbols) for all recipes – 2 principal axes purpose and interpretation. The 5 presented images correspond to the extremities of the map. White: gluten network labeled by Rhodamine B. Objective: $\times 40$ (water); Image = $220 \mu\text{m} \times 110 \mu\text{m}$ (115, Δ) 50% water, (108, 109) 55% water; (110, 111) 60% water. Water content is expressed in % as flour basis.

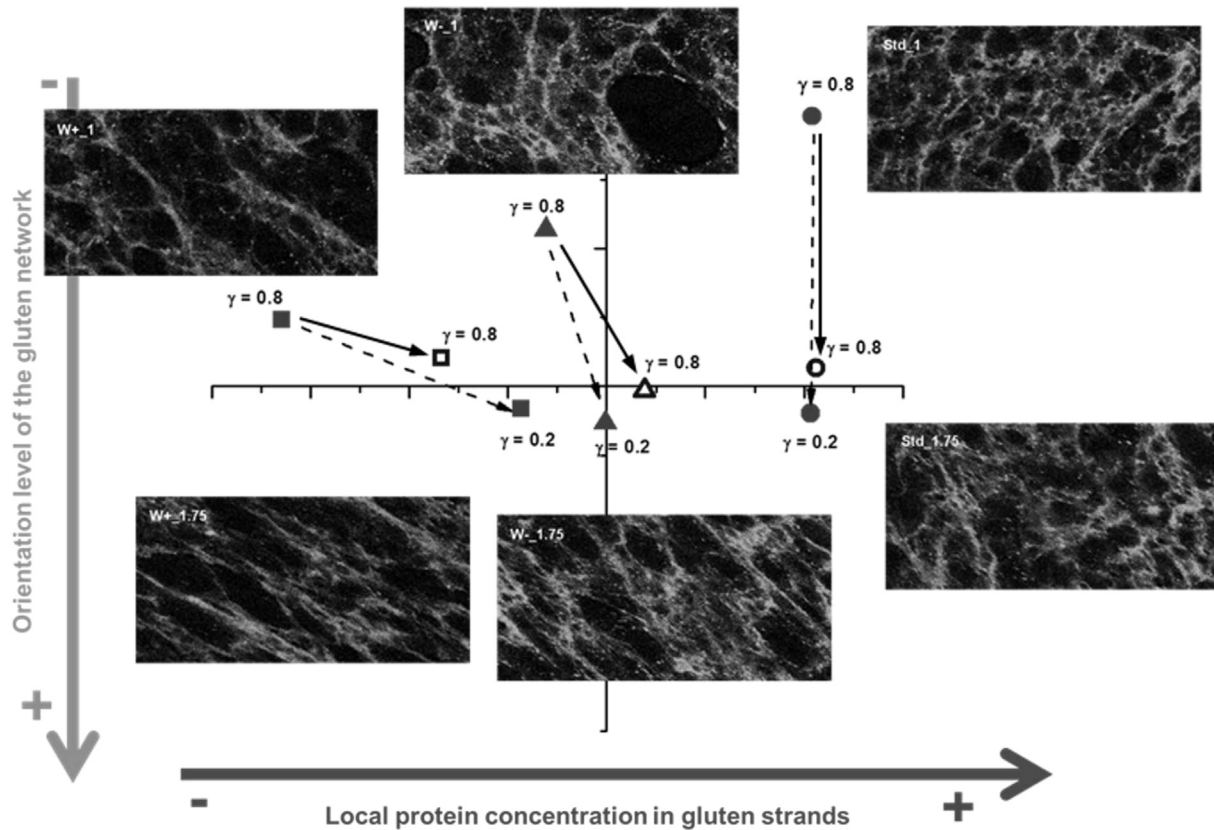


Fig. 5. PCA map with images of microstructures of wheat flour doughs for 2 different levels of deformation ($\gamma = 0.8$ and 0.2) in the range time of 1–2.5 s (filled symbols) and depending on the water content. Unfilled symbols correspond to $\gamma = 0.8$ in the range time of 28–29.75 s. White: gluten network labeled by Rhodamine B. Objective: $\times 40$ (water). Images = $220 \mu\text{m} \times 110 \mu\text{m}$ (115, Δ) 50% water, (108, 109) 55% water; (110, 111) 60% water. Water content are expressed in % as flour basis.

microstructures are compared depending on the composition. The 3 recipes did not have the same behavior.

For $\gamma = 0.8$, at the beginning of the oscillatory deformation, doughs can be classified according to the 2 principal components of the PCA. Std dough microstructure present a higher local protein concentration than W^- dough, itself more contrasted than W^+ dough. On the contrary, W^+ dough is a more oriented microstructure than W^- and Std doughs ones.

From $\gamma = 0.8$ to $\gamma = 0.2$, data shifted towards the vertical axis for Std, W^+ and W^- doughs and towards the horizontal axis for the W^+ dough and slightly for W^- doughs. For a high strain but no shear rate, network is not oriented whereas when shear rate is higher, due to the speed of the bottom plate, the gluten network adopted an orientation. The strands are elongated according to the shear rate level, mostly for Std and W^- doughs. Shearing increased slightly the local protein concentration in W^- dough. Finally, W^+ dough principal network modification is observed on the local protein concentration that increased when deformation level decreased. However, the gluten network was already more oriented than was the other doughs, and shearing lead to the same orientation level as the others.

For $\gamma = 0.2$, all images have the same y -coordinate which means that all doughs microstructure get the same orientation level. They have also the same order as for $\gamma = 0.8$ concerning the local protein concentration but with less differences in terms of protein content in microstructures.

3.3.3. Effect of the time of deformation

To illustrate the effect of the time of deformation, that is to say the effect of weariness, images at $\gamma = 0.8$, for $t = 1$ s and $t = 28$ s are considered (pink and blue symbols respectively, on Fig. 5). Whereas

at $t = 1$ s, images were distributed according to both PC, open symbols, corresponding to the same level of deformation 27 s later, are mostly spread along the PC1 but are at the same level of PC2. For all recipes, after 27 s of oscillation, a same level of orientation of the gluten network is attained. However, this level is much higher than the one observed at 1 s. The time of the deformation has given to the gluten network a more pronounced orientation. For W^+ and W^- , time of deformation has also an influence on the local protein concentration in the gluten strands, leading to an increase of this concentration with time. Actually, images at $\gamma = 0.8$ after 28 s of oscillation are close to the image at $\gamma = 0.2$ after 1.75 s. Changes in the microstructure have clearly happened due to the mechanical solicitation. However, doughs are ranked in the same order concerning the local protein concentration.

4. Conclusion

Using a rheo-optical device, observations of doughs microstructure under mechanical treatment were carried out. The present work has shown the potentiality of the device to follow the microstructure of consistent material such as wheat flour dough under conditions relevant with dough processing. Using confocal microscopy, several components can be studied when applied a thermo-mechanical treatment on a sample. As mechanical treatment are often responsible for the construction of the structure of a food product, coupling controlled deformation and mesoscopic observation allowed to illustrate and explain the rheological behavior of complex systems in terms of microstructures.

Concerning wheat flour doughs, qualitative differences in the behavior of the gluten network depending on the recipe and the

deformation level and time have been pointed out. Grayscale mathematical morphology, texture image analysis technique, was used to quantify and classify these differences. It was shown that the gluten network of the doughs could be ranked according to its local protein concentration and its level of orientation. Using this PCA map, one can classify different doughs depending on their recipe according to two principal informations: the local protein concentration and the orientation of the gluten network. The morphology of the gluten network and its evolution under shearing can help to understand the baking behavior. For instance, getting many and thin strands are better for baking compared to thick and few strands in the gluten network. In the latter one, continuous gluten network made of numerous thin strands, the network is less hermetic vs. the gas retention during proofing and baking. It will play a role on the final texture leading to a more regular and distributed crumb, in terms of alveolars.

These experiments provided promising data, qualitative and quantitative, concerning the weariness of the gluten network after being sheared at large amplitude oscillations along time. Results presented in this paper showed that using a complete methodology (observation under shearing + image texture analysis) on a realistic design of experiment would help to predict baking performance as well as sensory properties of the final product. Further work concerning the thermo-mechanical treatment of doughs will lead, for examples, to follow the growth of air bubbles within the network or to understand the influence of shearing on the final crumb of a bread. In addition, methods such as images analysis have to be set for quantifying these dynamic phenomenon and creating a direct link between properties and structure.

Acknowledgment

The authors wish to thank the MIMA2 Platform (INRA, Massy, France) for allowing us to use the CLSM. We are also grateful to Ile-de-France and Essonne for their financial support and the French Research Ministry for providing a PhD grant for these works.

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