Use of the 1H NMR technique to describe the kneading step of wholewheat dough: The effect of kneading time and total water content

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Abstract: The kneading step of wholewheat flour (WWF) dough was monitored using low-resolution 1H nuclear magnetic resonance (NMR). The tested variables were kneading time and total water content. Two 1H Free induction decay (FID) (A and B) and four 1H T2 Car-Purcell-Meiboom-Gill (CPMG) (C, D, E and F) proton populations were observed and the attribution to the different proton domains was made based on the literature and data acquisition. Kneading time significantly increased the mobility and the relative abundance of popA, the relative abundance and strength of protons of popC, D and E, while significantly reducing the relative amount of popF and increasing its mobility. This evolution of the proton populations during kneading was interpreted as chemical/physical transformations of the flour constituents. The use of WWF may reveal the changes in molecular dynamics underlying the higher water requirements of unrefined doughs, often associated with improved bread quality.

Cover Letter

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Dr. Paul Finglas
Editor-in-Chief
Food Chemistry

May 18, 2020

Dear Dr. Paul Finglas:

I am pleased to submit an original research article entitled "Use of the ^{1}H NMR technique to describe the kneading step of wholewheat dough: the effect of kneading time and total water content" for consideration for publication in *Food Chemistry.*

This manuscript applied the ^{1}H NMR technique to monitor the evolution of proton mobility and dynamics of a wholewheat flour dough (WWF) as a function of the kneading time and total water content. At first, the observed ^{1}H NMR dough signals were assigned to proton populations of flour biopolymers and water on the basis of the literature and data acquisition. The kneading time significantly affected the relative amount and mobility of the proton populations and the results were interpreted as physical/chemical transformations occurring to the main flour biopolymers (i.e. starch and gluten proteins) during the dough development. Significant differences in the proton distributions were also observed as a function of the total water content; the results revealed the importance of the proper flour hydration for the development of WWF dough.

We believe that this manuscript is appropriate for publication by *Food Chemistry* since for the first time the kneading step was described as the evolution of the ^{1}H NMR proton distributions. Our data showed that the ^{1}H NMR technique is able to monitor the chemical/physical phenomena occurring during the kneading step, hence encouraging its further application to correlate the dough molecular pattern to the physical characteristics of the final product. Furthermore, the significant differences of the proton distributions obtained as a function of the total water content may reveal the molecular reasons underlying the higher water requirements of unrefined wheat doughs often associated with a better quality of the final product.

This manuscript has not been published and is not under consideration for publication elsewhere. We have no conflicts of interest to disclose.

Thank you for your consideration!

Sincerely,

Dr. Lorenzo Guerrini
Department of Agricultural, Food and Forestry Systems Management (DAGRI)
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Responses to Reviewers
Manuscript FOODCHEM-D-20-04483

The authors would like to thank Reviewers, for the time spent in improving the paper and for the important suggestions and corrections proposed. We hope to have addressed all the issues that reviewers outlined.

Reviewers' comments:

Reviewer #1: The authors have addressed most of my comments in the manuscript. The texts on the axes in all the 5 figures as well as inside Figure 5 are overlapped and not clear. This issue should be fixed before publication.

We are grateful to the Reviewer for the observation, we did not notice the mistakes on Figure 5. The Manuscript now included the modified Figure 5 with the correct texts on the axes and inside the Figure.

Reviewer #3: All previously raised concerns are rectified.

Highlights

- The ^{1}H NMR technique monitored the kneading step of a wholewheat flour (WWF) dough
- The effect of the kneading time and the total water content was investigated
- The ^{1}H NMR signals were interpreted as biopolymer physical/chemical transformations
- Different water contents changed the proton mobility and dynamics of WWF doughs
- The ^{1}H NMR technique is able to monitor dough molecular evolution during kneading

Title

Use of the ^{1}H NMR technique to describe the kneading step of wholewheat dough: the effect of kneading time and total water content

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Abstract

The kneading step of wholewheat flour (WWF) dough was monitored using low-resolution ^{1}H nuclear magnetic resonance (NMR). The tested variables were kneading time and total water content. Two ^{1}H Free induction decay (FID) (A and B) and four ^{1}H T_2 Car-Purcell-Meiboom-Gill (CPMG) ~~proton populations~~ (C, D, E and F) proton populations were observed and the attribution to the different proton domains was made based on the literature and data acquisition. Kneading time significantly increased the mobility and the relative abundance of popA, the relative abundance and strength of protons of popC, D and E, while significantly reducing the relative amount of popF and increasing its mobility. This evolution of the proton populations during kneading was interpreted as chemical/physical transformations of the flour constituents. The use of WWF may reveal the changes in molecular dynamics underlying the higher water requirements of unrefined doughs, often associated with improved bread quality.

Keywords: unrefined flour, mixing step, wheat dough, molecular mobility

1. Introduction

Low-resolution (LR) proton nuclear magnetic resonance (^{1}H NMR) is a powerful, non-destructive technique that is used to evaluate food quality due to its ability to study the molecular mobility and dynamics of water and biopolymers during the processing and storage of foods (Kirtil, Cikrikci, Mccarthy, & Oztop, 2017).

In the literature, LR ^{1}H NMR analysis has been widely applied to cereal-based products, particularly ~~to investigate the complex phase transitions and phenomena that occur during the breadmaking and bread staling processes~~ to investigate the chemical and physical status of flour biopolymers and their interactions with water molecules in dough, bread, and flour model systems (Bosmans & Delcour, 2016). ~~The chemical/physical status of wheat flour polymers and the interactions between wheat flour polymers and water have been detected in flour model systems, dough and bread.~~ Studies on flour polymers have investigated ^{1}H NMR distributions of relaxation times in model systems, while trying to assign the different sample proton populations to protons of the main flour constituents, i.e., starch and gluten, and to water protons (Tang, Godward, & Hills, 2000; Tang, Brun, & Hills, 2001; Choi, & Kerr, 2003; Doona, & Baik, 2007; Bosmans, Lagrain, Deleu, Fierens, Hills, & Delcour, 2012). Some authors have applied the LR ^{1}H NMR technique to study wheat flour dough with different water contents and during simulated breadmaking conditions. These experiments have highlighted the molecular water dynamics and redistribution among biopolymers, as well as the physico-chemical transformations experienced by the biopolymers in wheat dough during heating and cooling, and during bread staling (Ruan, Wang, Chen, Fulcher, Pesheck, & Chakrabarti,1999; Kim, & Cornillon, 2001; Lopes Da Silva, Santos, Freitas, Brites, & Gil, 2007; Doona, & Baik, 2007; Lu, & Seetharaman, 2013; Rondeau-Mouro, Cambert, Kovrlija, Musse, Lucas, & Mariette, 2015; Nivelle, Beghin, Bosmans, & Delcour, 2019; Hopkins, Newling, Hucl, Scanlon, & Nickerson, 2019; Curti, Carini, Cobo, Bocher, & Vittadini, 2017). In this light, ^{1}H NMR has been also used to investigate the effect of the incorporation of milling by-products (bran and germ) on the proton mobility of both wheat doughs (Adams, Ragaee, & Abdel-aal, 2016; Hemdane, Jacobs, Bosmans, Verspreet, Delcour, & Courtin, 2017a,b; Li, Hou, Chen, Chung, & Gehring, 2014; Li, Liu, Wu, Wang, & Zhang, 2016; Lu, & Seetharaman, 2013; Wang, Ye, Li, Wei, Chen, & Zhao, 2017; Xiong, Zhang, Niu, & Zhao, 2017) and fresh and stored bread (Katina,

Salmenkallio-Marttila, Partanen, Forssell, & Autio, 2006; Curti, Carini, Bonacini, Tribuzio, & Vittadini, 2013; Hemdane, Jacobs, Bosmans, Verspreet, Delcour, & Courtin, 2017b).

However, despite the molecular insights on wholewheat dough and bread, and the correlations with their macroscopic properties shown in the literature, at the present time the use of unrefined flours in the breadmaking process is still an issue (Parenti, Guerrini, & Zanoni, 2020).

It is widely known that kneading is one of the most important phases in breadmaking. This stage enables the homogeneous mixing of all the ingredients, the hydration of the flour constituents, the phase transitions that involve proteins and amorphous starch, the development of the gluten network, and the inclusion of air bubbles, giving a viscoelastic dough as a result (Cuq, Yildiz, & Kokini, 2002; Zhou, Therdthai, & Hui, 2014). Kneading conditions significantly affect dough development and its rheological properties, the breadmaking performance and the quality of the final product (Zhou, Therdthai, & Hui, 2014); furthermore, flours with different degrees of refinement may require adapted kneading conditions and higher amounts of water than refined flours (Cappelli, Cini, Guerrini, Masella, Angeloni, & Parenti, 2019). Indeed, it is well known that the presence of the fibre fraction significantly changes the water redistribution during the entire breadmaking process. In the kneading step, competition for the water molecules may occur between the flour constituents and the fibre, which could negatively affect the gluten network formation (Hemdane, Jacobs, Bosmans, Verspreet, Delcour, & Courtin, 2017b).

To the best of the authors' knowledge, in the current literature there are very few studies that have applied the LR ^{1}H NMR technique to monitor proton mobility in wheat dough during the first step of the breadmaking process, i.e., kneading (Kim, & Cornillon, 2001; Sangpring, Fukuoka, Ban, Oishi, & Sakai, 2017). This research includes the study by Kim, & Cornillon (2001) who studied the molecular mobility of wheat doughs at the end of different kneading periods (3, 18 and 30 min) and during a heating treatment (from 30°C to 100°C). Furthermore, the work by Sangpring, Fukuoka, Ban, Oishi, & Sakai (2017) investigated the relationship between the mixing state of wheat flour dough and the mechanical energy generated using a vertical mixer, testing different revolution speeds for a total kneading time of 3 min (Sangpring, Fukuoka, Ban, Oishi, & Sakai, 2017).

In the present study, the ^{1}H NMR technique was applied to monitor the proton molecular dynamics and mobility in wholewheat flour (WWF) dough during the kneading step. Furthermore, due to the key role of the water amount in WWF dough, a comparison was made between the proton distributions obtained at two different dough moisture contents. A single pulse free

induction decay (FID) and Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence (^{1}H T_2 spin-spin relaxation) were applied to measure both fast relaxing and slowly relaxing protons. The kinetic evolution of the mobility and abundance of ^{1}H populations were monitored during the process so as to gain a new insight into the kneading phenomenon. The choice to use a WWF could improve the understanding of the molecular phenomena linked to the presence of milling by-products and could disclose new strategies for the development of processing conditions adapted in function of the characteristics of the raw material.

2. Materials and Methods

2.1 Materials

One batch of sp. *Triticum aestivum* L., cv. Verna WWF, was used to perform the experimental trial. The wheat was grown in Montespertoli (Florence, Italy) during the 2019-2020 growing season. The WWF was ground using a stone grinding mill and a sieve (two consecutive passages through a 1,100-1,200 µm sieve) at the Molino Paciscopi (Montespertoli, Florence, Italy). The flour belongs to the wholewheat category according to the Italian classification as the extraction rate and the ash content were in line with the standard benchmarks for this flour category (i.e., extraction rate 98 g/100 g dry kernel, ash content 1.3-1.7 g/100 g dm) (Zhou, Therdthai, & Hui, 2014). The mineral water (Sant'Anna, Vinadio, Italy) was purchased at a local market (Florence, Italy).

2.2 The experimental design

The ^{1}H NMR molecular mobility of WWF dough was studied using a full factorial design. The experimental trial tested the effect of two variables:

(i) The kneading time, i.e., *t*. Measurements were performed after every 3 min of kneading, from 3 to 24 min for a total of 8 measurements (t_1=3 min, t_2=6 min, t_3=9 min, t_4=12 min, t_5=15 min, t_6=18 min, t_7=21 min and t_8=24 min);

(ii) The total water content, i.e., *TW*, (%, g water/100 g flour). Two different levels were tested: 56% (w/flour w), i.e., the WWF56 samples, which corresponded to the amount of water to achieve a farinographic consistency of 500BU, vs 60% (w/flour w), i.e., the WWF60 samples.

2.3 Measurement methods

2.3.1 ^{1}H NMR measurements

Proton molecular mobility was investigated with a low-resolution (20 MHz) ^{1}H NMR spectrometer (the MiniSpec, Bruker Biospin, Milano, Italy) operating at 25.0 ± 0.1°C. ^{1}H free induction decay (FID) and ^{1}H T_2 Carr-Purcell-Meiboom-Gill (CPMG) experiments were used ~~– to reveal the respectively fastest and most slowly relaxing protons in the time frame of the experimental window~~. The FID experiment allows to detect very short relaxation time (in the range between 10-500 µs) which correspond to the less mobile protons in solid-like components and of protons of water molecules tightly associated with those of solids. Conversely, the more mobile protons (in the range between 0.1- 1000 ms) has to be detected with CMG pulse-sequence as the high relaxation times measured using FID sequence are not true spin–spin relaxation times because the FID signal contains also the lost signal due to local inhomogeneities in the magnetic field.

The dough ingredients were stored at room temperature (22 ± 2°C) and 500 g batches of dough were prepared; the basic formulation was: flour (310 g) and water (56% and 60% w/flour w). The dough was prepared at room temperature (22 ± 2°C) using a Kitchen Aid Professional Mixer (5KSM185PS, KitchenAid, St. Joseph, Michigan, USA) with a dough hook (model KSM35CDH), functioning at 110 rpm. Samples were analysed every 3 min during the kneading step for a total of 8 kneading periods (see above).

Due to the time required for the acquisition of the ^{1}H NMR signals and to the short time interval between the selected kneading points (3 min), and to ensure that all samples were analysed within a maximum of 1 minute after kneading (to avoid different resting times), two batches of dough had to be prepared for each replicate. Specifically, in order to be able to measure the 8 kneading times, the same dough replicate required the analysis of two different batches: in the first batch, the ^{1}H NMR parameters were acquired from each acquisition (t_3, t_9, t_{15}, t_{21}) after a 6-min interval; in the second batch, the complementary kneading points were analysed at the same time interval (t_6, t_{12}, t_{18}, t_{24}) in order to complete the ^{1}H NMR molecular kinetic of the dough. Therefore, variability was inevitably introduced to the data set due to the different dough mixing batches, since each dough replicate did not derive from the same sample and, as it is a complex food matrix, bread dough is known to have an intrinsically high level of variability. Hence, the experiments required the preparation of a total of 2 (dough samples) x 4 (replicates) x 2 (water levels) = 16 batches of dough.

Dough samples (approx. 4 g) were collected from the central part of the dough during kneading. These were quickly placed in 10 mm diameter NMR tubes, and tightly compressed to a height of 10.5 mm. The tubes were then sealed with Parafilm to prevent moisture loss during the experiment.

FIDs signals were acquired using a single 90° pulse, followed by a dwell time of 7 μs and a recycle delay of 1 s, a 0.5 ms acquisition window (the experimental window limit for ensuring the homogeneity of the magnetic field), 32 scans and 900 data points. Six ^{1}H FID replicates were acquired for each sample. A two-component (exponential and Gaussian) model was used to fit the curves in order to obtain quantitative information about the proton relaxation time and the percentage of protons belonging to the more rigid and more mobile proton populations measurable within the FID experimental time frame (7–500 μs). The FID curves were fitted using SigmaPlot v.6 software (Systat Software Inc., USA), according to the following equation:

$$f(x) = y0 + ae^{\left(-\frac{t}{TA}\right)} + ce^{\left(-\frac{t}{TB}\right)2} \quad [1]$$

where *y*0 is the intercept, *a* and *c* the relative abundance of populations A and B, and T_A and T_B the relaxation time of the relative populations.

^{1}H T_2 (transverse relaxation time) was obtained with a CPMG pulse sequence with a recycle delay of 1 s, an interpulse spacing of 0.04 ms, 2500 data points and 32 scans. In order to increase the signal-to-noise ratio, a high number of scans were applied. A high number of scans increases the temperature of the sample and a temperature equilibrium period is generally required before the next experiment. In this study it was not possible to wait an additional amount of time, as the dough resting time could have affected the ^{1}H T_2 signal. Thus, only one ^{1}H T_2 curve was acquired for each dough replicate, for a total of at least four replicates for each sample. This aspect strongly underlines the great capability of the experimental plane to represent ^{1}H dynamics and mobility in such a complex matrix as bread dough during kneading time. The ^{1}H T_2 curves were analysed as quasi-continuous distributions of relaxation times using UPEN software (Alma Mater Studiorum, Bologna, Italy). ^{1}H T_2 CPMG relaxation decays were also fitted with a discrete exponential model (Sigmaplot, v.6, Systat Software Inc., USA) in order to obtain relaxation times and proton population abundances, according to the following equation:

$$f(x) = y0 + ae^{-bx} + ce^{-dx} + ge^{-hx} + ie^{-fx} \quad [2]$$

where *y*0 is the intercept, *a*, *c*, *g* and *i* the relative abundance of populations C, D, E and F, and *b*, *d*, *h* and *f* the relaxation time of populations C, D, E and F.

2.4 Modelling and data processing

In order to predict the ^{1}H NMR parameters of the different water content dough samples (56% vs 60%) during kneading, the experimental data were fitted with a linear model for the continuous *t* variable and the categorical *TW* variable, and with a second-order model for the continuous variable *t*, according to the following equation:

$$y_{obs} = b_0 + b_1 t + b_2 TW + b_{12} tTW + b_{11} t^2 + b_{112} t^2 TW + \text{error} \quad [3]$$

where b_0 is a constant (the intercept); b_1 and b_2 represent the main effect of each factor (*t* and *TW*); b_{12} is the effect of the interaction between the first-order coefficient of the variables (*t***TW*); the square coefficient b_{11} reveals if the variable *t* gives a maximum or minimum within the experimental domain; and b_{112} represents the effect of the interaction between the second-order coefficient of *t* and *TW* (t^2**TW*).

The data were analysed with R software. A two-way ANOVA was performed in order to assess significant differences ($p < 0.05$) due to the tested variables (*t* and *TW*) and to their interaction (*t***TW*). The not significant terms ($p > 0.05$) were removed from the model as suggested by Dunn, & Smyth (2018). Following this, the model was further checked with the ANOVA model.

3. Results

3.1 ^{1}H NMR proton distributions

The FID experiment showed the presence of two proton populations which were named A (the less mobile ones) and B (the more mobile ones), relaxing in the range of 15.2-15.7 µs and 349.4-368.8 µs, respectively. The ^{1}H T_2 distributions of the relaxation times showed the presence of four populations identified as popC, popD, popE and popF, from the least to the most mobile proton population, respectively. The ^{1}H relaxation times were in the range of 0.29-0.50 ms, 3.25-4.04 ms, 10.03-15.03 ms and 41.84-53.76 ms for populations C, D, E and F, respectively. Considering the abundances of the ^{1}H populations, the relative abundance of population A + population B gives

100% of the FID proton signal, while the relative abundance of populations C, D, E and F gives 100% of the CPMG signal. The dominant FID population was population A, which encompassed 78.23-80.17% of the total observable protons (population B represented 19.83-21.77% of the total protons). In the ^{1}H T_2 time frame window, the dominant population was population E, representing 52.57-56.79% of the total detectable protons, followed by population D (25.56-29.91%), population C (8.26-10.49%) and population F (5.40-14.60%). Since the relaxation times of populations B and C overlapped, these proton populations were considered to represent the same protons and therefore only population C was discussed as belonging to the better resolved CPMG experiment signal. As further confirmation of this hypothesis, the relaxation time of populations B and C showed the same results in function of the tested variables.

3.2 The effect of the kneading time

Kneading time, i.e., the variable *t*, significantly affected the ^{1}H NMR distributions.

Considering the FID signal, the results showed that *t* significantly impacted the relative abundance of population A (p=0.0006391) and its relaxation time, T_A (p=0.02770). The relative abundance of population A showed a linear increase of approx. 0.3% during the kneading step, from 79.39% to 79.68%; both the samples are represented as parallel straight lines with a positive and constant slope (Fig. 1a). In a similar manner to the relative abundance of population A, T_A showed a significant and linear increase during the kneading time, represented in Fig. 1b by the positive slope that characterised the trend of the parameter in the tested samples.

Considering the $^{1}HT_2$ results, a significant main effect of the kneading time was observed on the relative abundance of population C (t: p=2.878 10^{-8}), D (t: p=3.604 10^{-7}) and E (t: p<2.2 10^{-16}; t^2: p=4.066 10^{-8}) and on their relaxation times T_{2C} (t: p=0.002413), T_{2D} (t: p=4.413 10^{-8}) and T_{2E} (t: p=6.915 10^{-8}). These parameters showed a similar trend: an increase in the relative abundances and a simultaneous decrease in the relaxation times (Figs. 2a,b,c and Figs. 3a,b,c).

The relative abundance of populations C and D was significantly impacted by the first-order coefficient of *t*: the parameters showed a linear increase during the kneading time. The relative abundance of population C grew from 8.85% to 9.60%, whereas that of population D was affected by the interaction *t*TW*, hence it is discussed in the next paragraph. This effect can be graphically observed in the positive slope of both parameters in the tested samples throughout the process (Figs. 2a,b). With regard to T_{2C} and T_{2D}, the results showed a linear decrease in both parameters throughout the process, graphically represented by the negative constant slope that characterised

256 the trend of these relaxation times in the tested batches of dough (Figs. 3a,b). Specifically, T_{2C}
257 decreased from a mean value of 0.33 ms to 0.27 ms, and T_{2D} reduced from 3.67 ms to 3.43 ms.
258 The relative abundance of population E was affected by the first- and second-order coefficient of
259 the kneading time (i.e., *t* and t^2) whereas its relaxation time T_{2E} was significantly impacted only by
260 the first-order coefficient of *t*. This means that the relative abundance of population E increased as
261 a parabolic curve, revealing a greater rise at the beginning of the process followed by a lower
262 increase (Fig. 2c). Conversely, T_{2E} showed a linear downward trend, decreasing from 14.43 ms to
263 13.15 ms (Fig. 3c).
264 The relative abundance of population F was significantly affected by *t* and t^2 ($p<2.2\ 10^{-16}$ and
265 $p=0.003156$, respectively) and its relaxation time T_{2F} by *t* ($p<2.2\ 10^{-16}$). These parameters showed
266 the opposite behaviour compared to the proton distributions C, D and E. The relative abundance
267 of population F showed a parabolic reduction throughout the kneading step (from 11.26% to
268 6.77%) (Fig. 2d), whereas T_{2F} revealed an upward linear trend (increasing from 43.88 ms to 49.86
269 ms) (Fig. 3d).
270
271 *3.3 The effect of total water content*
272 The total water content of the dough, i.e., the variable *TW*, significantly affected the ^{1}H NMR
273 distributions. The main effect of *TW* showed similar results on the relative abundance of
274 populations A ($p=9.068\ 10^{-12}$), C ($p=0.0003139$) and D ($p=3.487\ 10^{-16}$): the WWF56 samples were
275 characterised by a higher relative abundance of these proton populations than the WWF60
276 samples. This effect can be graphically observed by the higher value of the intercept in the batches
277 of dough with the lower water content (WWF56) compared to the ones with the higher water
278 content (WWF60) (Figs. 1a and 2a, b). The values of the WWF56 samples compared to the values
279 of the WWF60 samples were 79.75% *vs* 79.25%, and 9.33% *vs* 9.09%, for populations A and C,
280 respectively. The relative abundance of population D was also affected by the interaction *t*TW*
281 ($p=0.04557$) and it showed a higher increase in the WWF56 samples than in the WWF60 batches
282 of dough during kneading. Specifically, the values of the WWF56 samples compared to the
283 WWF60 samples at the beginning and at the end of the kneading step were 27.36% *vs* 26.20% and
284 29.28% *vs* 27.12%, respectively. In Fig. 2b this effect is represented by the steeper slope of the
285 trend of the parameter in the batches of dough with the lower water content (WWF56) compared
286 to those with the higher water content (WWF60) during kneading.

The effect of *TW* on the relative abundances of populations E (p=0.0001011) and F (p=1.735 10^{-10}) was the opposite compared to populations A, C and D: the WWF60 samples were characterised by higher values of both parameters compared to the WWF56 samples. Indeed, the batches of dough with the higher water content (WWF60) revealed a greater intercept value compared to the samples with the lower water content (WWF56) (Figs. 2c, d). The relative abundance of population F was 8.98% in the WWF60 samples and 7.96% in the WWF56 samples. The relative abundance of population E was also affected by the interaction *TW***t* (p=0.0155801): the WWF60 samples were characterised by a higher increase in the parameter during the kneading step than the WWF56 samples. At the beginning of the process, the value of the parameter was similar for the two different water contents: the WWF56 samples showed a value of 53.02% and in the WWF60 samples the parameter was 53.22%. However, during kneading, the two samples showed a different trend in the parameter in function of the total water content: in the WWF60 samples the relative amount of population E continued increasing up to 56.08% (21 min), whereas in the WWF56 samples the parameter reached a maximum at lower values (54.78%) (at approx. 18 min). This effect is graphically represented by the steeper slope of the trend of the parameter in the WWF60 batches of dough compared to the WWF56 batches of dough (Fig. 2c)

Considering the significant effect of *TW* on the relaxation times of the proton distributions, the results highlighted that the WWF60 samples were characterised by a significant increase in T_{2E} (p=9.614 10^{-5}) compared to the WWF56 samples, whereas the opposite effect was obtained for T_{2C} (p=0.004712). These effects can be graphically observed in Fig. 1b and Fig. 3c: the trend of T_{2E} showed a greater intercept in the batches of dough with the higher water content (WWF60) than in the ones with the lower water content (WWF56), while T_{2C} showed a greater intercept in the WWF56 compared to the WWF60 batches of dough. The T_{2E} values were 13.84 ms in the WWF60 samples and 13.39 ms in the WWF56 samples. Conversely, the T_{2C} values were significantly lower in the WWF60 samples than in the WWF56 samples, at 0.29 ms and 0.33 ms, respectively.

4. Discussion

The present study applied the LR ^{1}H NMR technique to describe the proton distributions and dynamics of a WWF dough during the kneading step of breadmaking process. Scant information is given in the literature on the molecular proton changes during dough kneading (Kim, & Cornillon, 2001; Sangpring, Fukuoka, Ban, Oishi, & Sakai, 2017). Indeed, while Kim, & Cornillon (2001) investigated ^{1}H NMR molecular mobility on dough produced using three different final mixing

times (i.e., 3, 18 and 30 min), Sangpring, Fukuoka, Ban, Oishi, & Sakai (2017) only monitored the first 3 min of the kneading step.

The theoretical state diagram showing the physical changes as a function of temperature and water content during breadmaking can aid the discussion and interpretation of the ^{1}H molecular signal detected in the kneading step (Cuq, Yildiz, & Kokini, 2002). The first two stages of the theoretical state diagram represent the phenomena associated with the kneading step: the full hydration of the flour constituents and the mechanical energy input produced by the mixer (Cuq, Yildiz, & Kokini, 2002). The resultant effects of the hydration and mechanical energy input include (i) swelling of the starch granules and glass transition in amorphous regions of the semi-crystalline starch structure; (ii) glass transition of the proteins and their interactions through cross-links promoted by the increase in molecular mobility (Cuq, Yildiz, & Kokini, 2002). Moreover, in a study of the rheology of wheat flour dough during the kneading step (Gras, Carpenter, & Anderssen, 2000), the evolving rheological properties of the dough in effect monitored the molecular processes in terms of bounded and unbounded water within the flour constituents occurring during mixing.

Although we studied a WWF dough system, since the main constituents of WWF are the starch and protein components, and since in the literature the presence of milling by-products in WWF dough is not seen to significantly modify the relaxation times of the proton populations (Hemdane, Jacobs, Bosmans, Verspreet, Delcour, & Courtin, 2017b), at first we discuss the proton attributions based on the most relevant reference (Bosmans, Lagrain, Deleu, Fierens, Hills, & Delcour, 2012), then we focus on the influence of the milling by-products (Hemdane, Jacobs, Bosmans, Verspreet, Delcour, & Courtin, 2017b).

4.1 Proton attributions and the effect of the kneading time on ^{1}H NMR kinetics

In this study, the kneading time significantly affected all the NMR parameters detected in the FID and CPMG experiments. The most rigid and abundant FID population (population A), showed a slight but significant increase in its relative amount and mobility during kneading (Fig.1 a,b). According to the literature (Bosmans, Lagrain, Deleu, Fierens, Hills, & Delcour, 2012), this protons population is assigned to the CH protons of crystalline starch, amorphous starch and gluten not in contact with water. Since starch is the main component of the WWF dough system, it could be hypothesised that the changes in population A mainly reflected the changes in the starch structures during kneading. It is known that at room temperature and in presence of a sufficient

amount of water, starch granules swell to a limited extent, adsorbing up to 46% of their dry weight of water (Goesaert, Brijs, Veraverbeke, Courtin, Gebruers, & Delcour, 2005). As a result, the volume and the surface of the starch granules grow, probably causing an increase in the starch protons exposed on the granule surface. Furthermore, the starch hydration is responsible to the glass transition of the amorphous regions which increase their molecular mobility. The significant rise in the relative amount of population A (Fig.1 a) could be interpreted as the effect of the hydration of the starch which caused the granules to swell. Instead, the significant rise in the mobility of population A (Fig.1 b) may be related to the increased mobility of the starch amorphous chains due to the glass transition as a result of kneading. ~~swelling of the starch granules and the glass transition of the amorphous starch regions may have led to the increase in the structural molecular mobility, causing a shift in the relaxation time of these protons towards higher values.~~

Population E was the dominant population in the CPMG proton distribution, and it showed significant changes during the kneading step (Fig. 4). Population E was assigned to the overlapped populations of starch extra-granular water and water in the gluten matrix, including mobile protons of water in exchange with hydroxyl protons of starch on the granule surface, and to water protons surrounding the sheets in exchange with gluten protons, in accordance with Bosmans, Lagrain, Deleu, Fierens, Hills, & Delcour (2012). In the literature, proton relaxing in a comparable relaxation time range of population E are the protons associated with the greatest changes during the different phases of dough processing, showing differences in function of the water content of the dough (Ruan, Wang, Chen, Fulcher, Pesheck, & Chakrabarti,1999; Doona, & Baik, 2007; Lu, & Seetharaman, 2013; Hemdane, Jacobs, Bosmans, Verspreet, Delcour, & Courtin 2017a), storage conditions and presence of fibres (Lu, & Seetharaman, 2013), and different bread production methods (Li, Deng, Li, Liu, & Bian, 2015), as well as during the whole breadmaking process (Nivelle, Beghin, Bosmans, & Delcour, 2019). In our case too, significant changes in the relative abundance of population E were observed during the kneading time (Fig.2 c, Fig.4). At the beginning of the process, the relative abundance of population E grew at a faster rate, which could be associated with the more mobile water protons that progressively bound to the gluten proteins during the hydration phase (Fig.2 c). Indeed, in the literature data, it is reported that the dominant phase formed during hydration is represented by the water binding to the proteins (Gras, Carpenter, & Anderssen, 2000). As a consequence of the hydration of the proteins, they transited from a glassy to a rubbery state (Cuq, Yildiz, & Kokini, 2002). After the initial fast increase, the relative amount

383 of population E showed a parabolic trend, coming close to a peak in the last stages of the process
384 (Fig.2 c). This behaviour could be associated with the phenomena occurring after the gluten
385 proteins have adsorbed a sufficient amount of water: the development of the gluten network
386 through cross-linking interactions. The trend shown by the relaxation time of population E further
387 confirmed this hypothesis (Fig. 3c). The parameter significantly decreased during kneading,
388 revealing that the water protons of population E became progressively more tightly bound to the
389 flour constituents, primarily represented by the gluten proteins. Furthermore, the constant
390 decrease in the parameter after the initial hydration phase could be ascribed to the development
391 of the gluten network, which enhances the molecular organisation, possibly explaining the shift in
392 the relaxation time towards lower values. The NMR parameters of populations C and D changed in
393 a linear manner during kneading: there was a significant increase in the relative amount of
394 protons with a concurrent significant reduction in their relaxation time (Fig.2 a,b; Fig3 a,b). The
395 increase in the relative amounts of these proton distributions was characterised by a lower growth
396 and followed a linear trend unlike the parabolic trend of population E. According to the research
397 by Bosmans, Lagrain, Deleu, Fierens, Hills, & Delcour (2012), these populations were assigned to
398 some CH protons of amorphous starch and CH protons of gluten in the sheets with little contact
399 with the confined water (population C) and to hydroxyl protons of intra-granular water and starch.
400 In addition, they were also assigned to some CH protons of gluten and exchanging protons of
401 confined water and gluten (population D). Hence, our results showed that, during the kneading
402 step, the water protons bound to populations C and D increased and became more strongly bound
403 to the flour constituents.
404 The most mobile CPMG population, population F, revealed marked changes in the NMR
405 parameters during the process (Fig.2 d; Fig.3 d). Interestingly, the trend observed for this
406 population was exactly the opposite to that of population E. Indeed, the relative amount of
407 population F showed a decreasing parabolic trend: in the initial phase of the kneading, the
408 parameter showed a greater decrease, whereas in the last phases it reduced to a smaller extent
409 (Fig.2 d). In the literature, the most mobile proton population, identified as population F in the
410 present study, has been differently attributed. Hemdane, Jacobs, Bosmans, Verspreet, Delcour, &
411 Courtin (2017a), who studied proton molecular dynamics and distributions in wheat flour dough
412 and milling by-products, and Hemdane, Jacobs, Bosmans, Verspreet, Delcour, & Courtin (2017b),
413 who investigated ^{1}H populations in bran-enriched doughs compared to refined doughs, assigned
414 the most mobile population to the water protons bound to the flour lipids present in the bran and

415 germ fractions (relaxation time of approx. 100 ms). On the other hand, Assifaoui, Champion,
416 Chiotelli, & Verel (2006) and Serial et al. (2016) attributed the most mobile population detected in
417 biscuit dough systems to the lipids in the biscuit formula (relaxation time of 100 ms and 100-1000
418 ms, respectively). Conversely, Lu, & Seetharaman (2013), Li, Deng, Li, Liu, & Bian (2015) and Wang,
419 Ye, Li, Wei, Chen, & Zhao (2017), who studied wheat doughs from refined and fibre-enriched
420 flours, attributed the most mobile proton population to weakly bound protons of water
421 (relaxation time of 100 ms, 30-100 ms and 37-115 ms, respectively). Our results (relaxation time of
422 population F and its trend during the kneading step) are consistent with what is reported by Lu, &
423 Seetharaman (2013), Li, Deng, Li, Liu, & Bian (2015) and Wang, Ye, Li, Wei, Chen, & Zhao (2017),
424 supporting the hypothesis that this proton distribution corresponds to weakly bound protons of
425 water. Indeed, at the beginning of the kneading, before the hydration of the flour occurred, this
426 proton population showed the highest relative abundance as compared to final kneading times
427 (Fig.2 d). Hence, it could be hypothesised that the protons of population F progressively bound to
428 the flour constituents, mainly those of population E, followed by populations C and D, as shown by
429 the significant increase in their relative amounts. Indeed, Wesley, Larsen, Osborne, & Skerritt
430 (1998) reported that unbound water decreased quite rapidly during the hydration phase. As
431 further confirmation, the sum of the rise in the relative amount of populations C, D and E
432 corresponded approximately to the reduction of the relative amount of population F. The
433 relaxation time of population F showed the opposite trend to the other CPMG populations: it
434 increased linearly during the kneading step, revealing that these water protons became
435 progressively more mobile (Fig.3 d). This means that weakly bound protons of water become
436 progressively not only less abundant but also more mobile during kneading. This weakly bound
437 fraction of water that is retained at the end of the kneading phase could have an important role in
438 the plasticization of the dough structure. A schematic representation of the authors'
439 interpretation of ^{1}H NMR results as a function of the kneading time is reported in Fig. 5.
440 Our WWF dough system contained both the bran and the germ fractions, which contributed to the
441 observed ^{1}H NMR signals. The literature contains scant information on the proton molecular
442 dynamics and mobility in a similar dough system (Hemdane, Jacobs, Bosmans, Verspreet, Delcour,
443 & Courtin, 2017b). Hemdane, Jacobs, Bosmans, Verspreet, Delcour, & Courtin (2017b) have
444 studied the influence of the bran fractions on water mobility and biopolymer behaviour during
445 breadmaking and storage. Their results showed that FID and CPMG proton populations in the
446 bran-enriched dough after the mixing step relaxed in the same relaxation times range as

populations found in the refined dough sample used as a control (Hemdane, Jacobs, Bosmans, Verspreet, Delcour, & Courtin, 2017b). The main observed differences were the relative abundance of the proton populations: popA was approx. 16-20% less abundant in the bran-enriched dough, and this effect was related to the greater amount of water required by these doughs compared to the control sample (Hemdane, Jacobs, Bosmans, Verspreet, Delcour, & Courtin, 2017b). The CPMG signal revealed a higher relative amount of popC, E and F in the bran-enriched doughs and a greater relative abundance of popD in the control sample (Hemdane, Jacobs, Bosmans, Verspreet, Delcour, & Courtin, 2017b). The different proton distributions in the bran-enriched dough can be assigned to CH protons of amorphous starch and dietary fibre constituents such as arabinoxylan (popC), exchanging protons of bran- and flour- related biopolymers and water interacting with these biopolymers outside the starch granules (popE), and protons originating from lipids (popF) (Hemdane, Jacobs, Bosmans, Verspreet, Delcour, & Courtin, 2017b). The higher popD observed in the refined sample was assigned to CH protons of gluten and exchanging protons of gluten and of starch, and water inside the starch granules (Hemdane, Jacobs, Bosmans, Verspreet, Delcour, & Courtin, 2017b). Hence, in our WWF dough samples the presence of milling by-products, although in a smaller amount compared to the main flour constituents, could have contributed to the characteristic ^{1}H NMR profile. Furthermore, since the bran showed a high water-binding capacity (Hemdane, Jacobs, Bosmans, Verspreet, Delcour, & Courtin, 2017a,b), it could have impacted the results obtained as a function of the total water content.

4.2 The effect of total water content on ^{1}H NMR kinetics

The total water content of the dough significantly affected the NMR parameters. The two levels of water tested were 56% and 60% (w/flour w) which corresponded to a total dough hydration of 43.65% and 45.06%, respectively. The batches of dough with a 56% water content were characterised by a higher relative amount of populations A, C and D (Fig.1 a; Fig.2 a,b), a higher relaxation time of population C (Fig.3 a), and a greater increase in the mobility of population A (Fig.1 b) and in the relative amount of population D during kneading (Fig.3 b). Conversely, the batches of dough with a 60% water content showed a higher relative abundance of populations E and F (Fig.2 c,d), a greater relaxation time of the prevalent populations A and E (Fig.1 b; Fig.3 c), and a higher increase in population E during kneading (Fig.2 c).

These results revealed that a change in the total dough water content of approx. 1.4% produced significant differences among the proton distributions within the dough system. Several studies have investigated the effect of different dough water contents on ^{1}H NMR molecular dynamics and mobility (Ruan, Wang, Chen, Fulcher, Pesheck, & Chakrabarti,1999; Choi, & Kerr, 2003; Wang, Choi, & Kerr, 2004; Assifaoui, Champion, Chiotelli, & Verel, 2006; Doona, & Baik, 2007; Lu, & Seetharaman, 2013). However, to the best of the authors' knowledge no studies have tested the effect of the dough water content during the kneading step.

Similar results showing a decrease in the relative amount of the less mobile detected population (approximately our popA) alongside an increase in the dough water content have been reported in the literature and associated with the complete hydration of all the water-binding sites on the flour solids (Ruan, Wang, Chen, Fulcher, Pesheck, & Chakrabarti,1999; Choi, & Kerr, 2003; Doona, & Baik, 2007; Hemdane, Jacobs, Bosmans, Verspreet, Delcour, & Courtin, 2017b).

The effects observed in the present study on CPMG proton distributions indicated that the total water content produced significant differences in the redistribution pattern of the water molecules among the flour constituents. The higher the water availability (60% batches of dough), the more the ~~water~~ protons belonging to the more mobile proton populations E and F and the higher the mobility of population E. Furthermore, the mobility of ~~population E increased, whereas that of~~ population C decreased. Figure 2c clearly shows that the relative amount of population E peaked at different kneading times in function of the total water content: the less hydrated dough (56% batches of dough) reached the maximum value of the parameter at approx. minute 18 of the kneading step. Conversely, in the more hydrated sample (60% batches of dough), the relative abundance of population E continued to increase throughout the process, reaching maximum level at minute 21 of the process, at a higher value than what was observed in the less hydrated dough (Figure 2c). This result can be also observed in Figure 4, showing the representative proton T_2 distributions of the WWF dough samples obtained at two different kneading times: (i) after 6 minutes of the kneading step for both 56% and 60% water doughs and (ii) at the kneading time corresponding to the highest relative amount of popE (i.e. 18 min in 56% water doughs, 20 min in 60% water doughs). In the literature, it is known that the farinographic test cannot correctly predict the water absorption of WWF, and usually a higher water content is required to improve product quality (Bruckner, Habernicht, Carlson, Wichman, & Talbert, 2001; Hemdane, Jacobs, Dornez, Verspreet, Delcour, & Courtin, 2016). Indeed, it has been found that the presence of fibres not only causes a dilution effect on the gluten proteins, but fibres also have a strong water binding

capacity that negatively affects dough development owing to several phenomena which are not yet fully understood (Hemdane, Jacobs, Bosmans, Verspreet, Delcour, & Courtin, 2017b).

Our results disclosed that in WWF dough systems a higher water content produces a greater hydration of the biopolymers belonging to population E. This data may highlight that a higher water availability produced a better hydration of the gluten proteins by reducing their competition for water molecules with the bran fractions relaxing in this relaxation time range (Hemdane, Jacobs, Bosmans, Verspreet, Delcour, & Courtin, 2017b). Furthermore, a higher amount of weakly bound water (population F) was observed in WWF doughs at the higher water content. Both these effects may account for the positive role of greater hydration on WWF dough, which is generally associated with an improvement in the flour's technological properties and bread quality (Bruckner, Habernicht, Carlson, Wichman, & Talbert, 2001; Hemdane, Jacobs, Dornez, Verspreet, Delcour, & Courtin, 2016). Furthermore, it can also be speculated that the time required for the WWF system to reach full hydration is longer when a higher amount of water is available, as suggested by the different maximum values shown by the dough samples (18 min and 21 min, respectively).

5. Conclusions

In this study, ^{1}H NMR was applied for the first time to monitor the proton mobility and dynamics in wholewheat flour ~~(WWF)~~ based dough, as a function of the kneading time and total water content. ~~Two faster relaxing proton populations were detected within the time frame of the FID experimental window and four slower relaxing populations within the time frame of the CPMG experimental window. According to the literature and data reported in the present study, these are related to the protons of the flour constituents as well as to water bound to biopolymers.~~

~~The kneading time significantly affected the proton distributions of the WWF dough. The most significant molecular changes concerned populations A, E and F, and were related to the chemical/physical phenomena occurring during the kneading step. The significant rise in the relative abundance and mobility of population A was interpreted as the swelling of the starch granules and the glass transition of the amorphous starch regions. The significant increase in the relative amount of population E was interpreted as the progressive hydration of the gluten proteins by the most mobile water protons, followed by the development of the gluten matrix. This hypothesis was further confirmed by the decreasing trend in the mobility of population E. The reduction in the relative amount and the concomitant increase in mobility of the most mobile~~

~~population (population F) may disclose that this population consisted of weakly bound water that progressively hydrated the flour constituents during kneading.~~

A significant effect of the kneading time was observed on all ^{1}H NMR parameters and results were interpreted as physical/chemical phenomena occurring during kneading. The protons belonging to the less mobile population (population A) and to the two most mobile populations, (population E and F) showed the major changes, interpreted in terms of starch granules swelling, glass transition of amorphous starch regions, proteins hydration and gluten formation, and gradual decrease of free water during kneading.

The effect of total water content may have revealed the molecular insights making the use of high water amounts a key factor for WWF based products. The increase in the total water content led to a significant increase of the relative abundance of the most mobile populations (i.e., E and F), and to a growth in the mobility of populations A and E. The higher hydration of gluten proteins and higher free water fraction may be responsible for the general improvement of WWF dough and bread properties when higher water amounts than that predicted by the Farinograph are used. ~~These results could reveal the molecular reasons underlying the higher water requirements of WWF dough compared to more refined dough. Moreover, the higher amounts of population E and F in sample with the higher water content may be associated with the improved technological properties and better quality of WWF products containing a higher amount of water than predicted by the farinograph.~~ However, other experiments are required to confirm these hypotheses. In this light, the use of ^{1}H NMR may help to better understand the molecular dynamics within the dough system so as to discover innovative processing strategies specifically adapted to the different characteristics of WWF.

This first study of the use of ^{1}H NMR to monitor the kneading step of breadmaking has shown that this technique is able to detect the main chemical/physical phenomena occurring during kneading. This encourages its further application in order to correlate the molecular pattern of dough to the physical characteristics of the final product.

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***Declaration of Interest Statement**

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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Figure(s)

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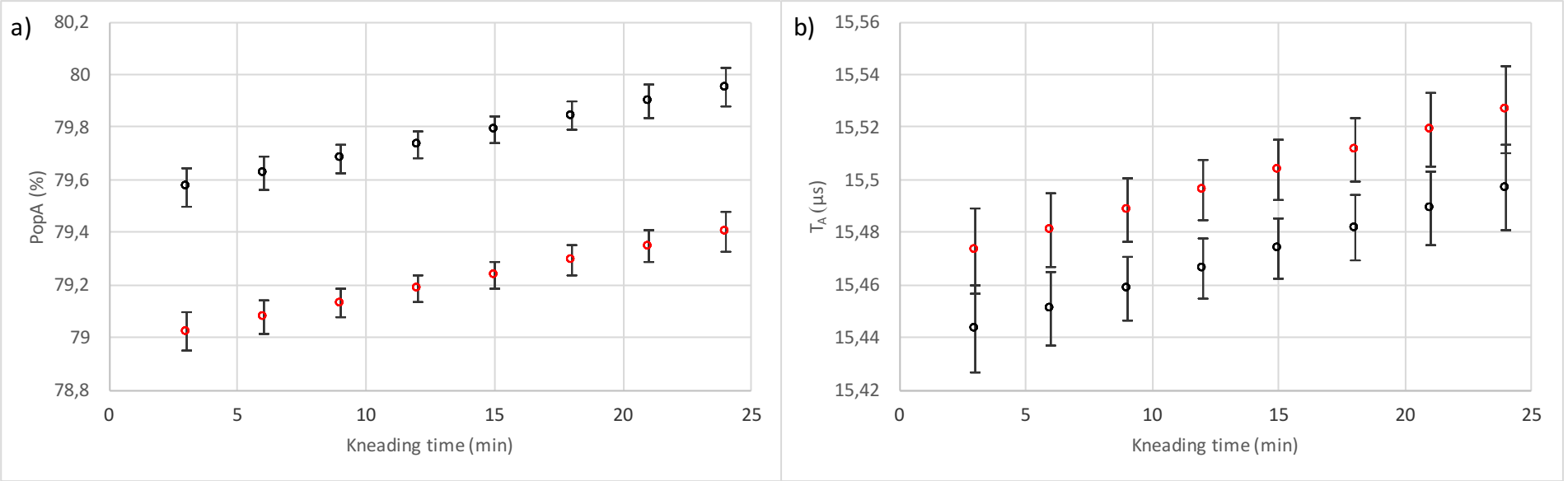

Figure 1 Kinetic models of ^{1}H NMR proton population in WWF doughs during the kneading process obtained from the experimental data of single pulse Free Induction Decay (FID): a) the relative abundance of population A, popA (%), and b) the relaxation time of population A, T_A (µs). Symbol "o" represents WWF56 (WWF dough at 56% of water content), and "o" WWF60 (WWF dough at 60% of water content). Black bars represent the 95% confidence interval of the model.

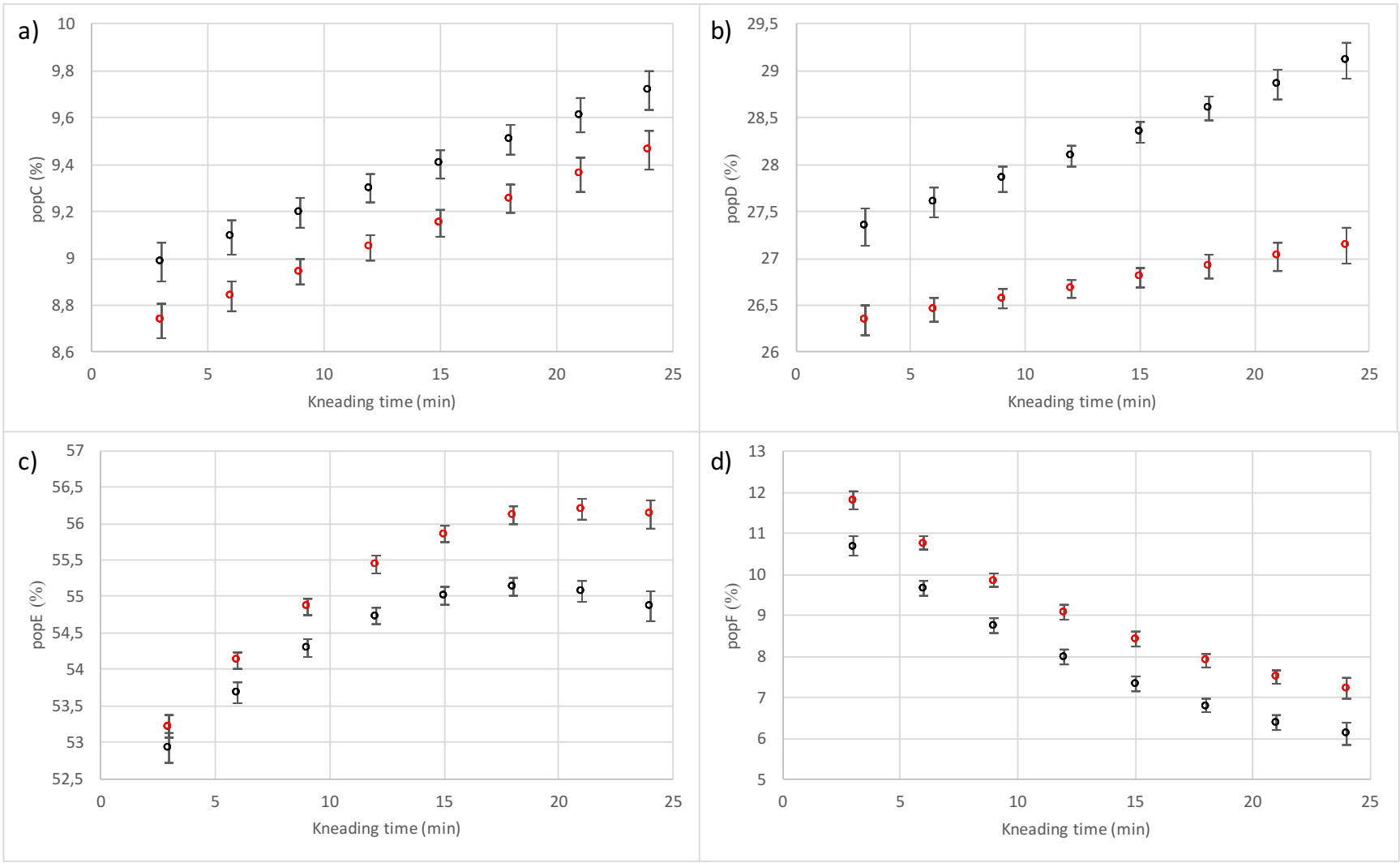

Figure 2 Kinetic models of ^{1}H NMR proton populations in WWF doughs during the kneading process obtained from the experimental data of Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence: a) the relative abundance of population C, popC (%), b) the relative abundance of population D, popD (%), c) the relative abundance of population E, popE (%), d) the relative abundance of population F, popF (%). Symbol "o" represents WWF56 (WWF dough at 56% of water content), and "o" WWF60 (WWF dough at 60% of water content). Black bars represent the 95% confidence interval of the model.

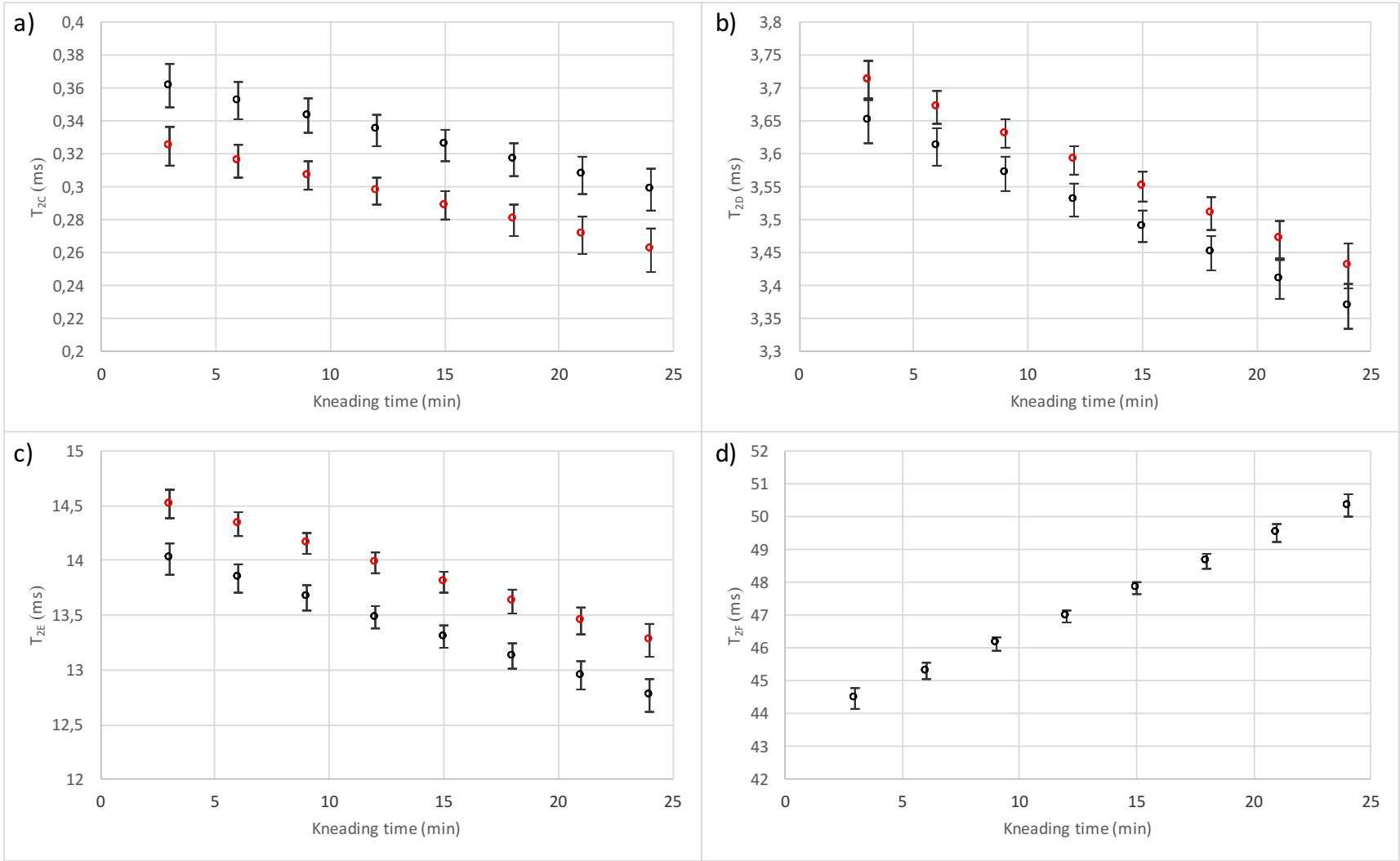

Figure 3 Kinetic models of ^{1}H NMR proton distributions in WWF doughs during the kneading process obtained from the experimental data of Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence: a) the relaxation time of population C, T_{2C} (ms), b) the relaxation time of population D, T_{2D} (ms), c) the relaxation time of population E, T_{2E} (ms), d) the relaxation time of population F, T_{2F} (ms). Symbol "o" represents WWF56 (WWF dough at 56% of water content), and "o" WWF60 (WWF dough at 60% of water content). In graph "d" the tested doughs overlapped and are both represented as "o". Black bars represent the 95% confidence interval of the model.

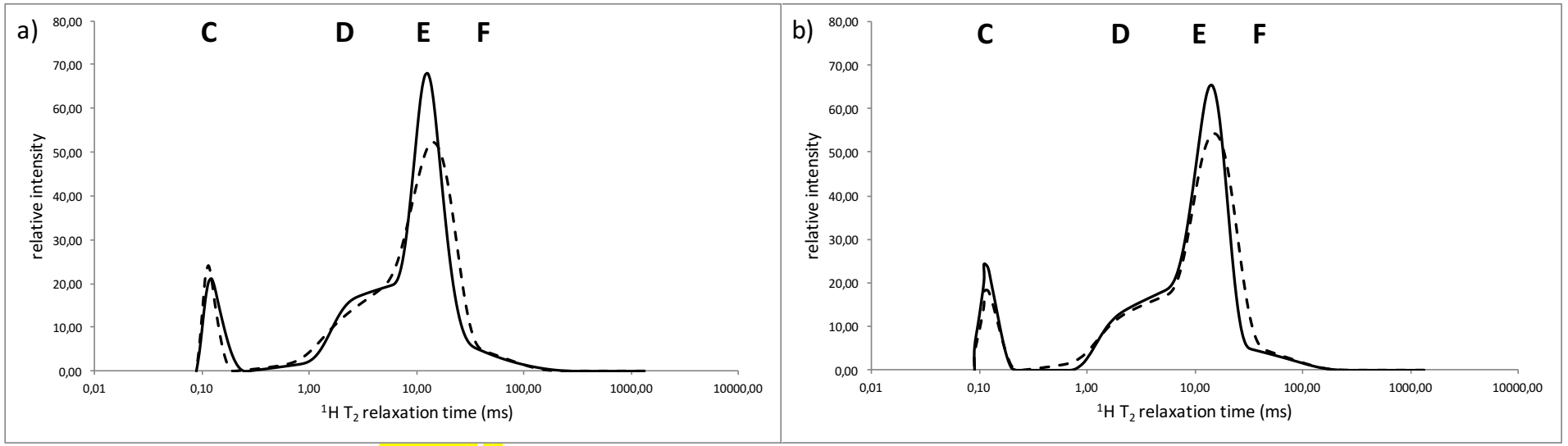

Figure 4 Representative CPMG proton ~~molecular~~ T_2 distributions of the WWF dough samples during the kneading step: a) WWF dough samples containing 56% of water content (the WWF56 samples), b) WWF dough samples containing 60% of water content (the WWF60 samples). Dashed lines represent the dough samples after 6 min of the kneading step and solid lines the dough samples at the kneading time where the highest amount the dominant population (popE) was reached (t=18 min in the WWF56 samples, t=21 min in the WWF60 samples).

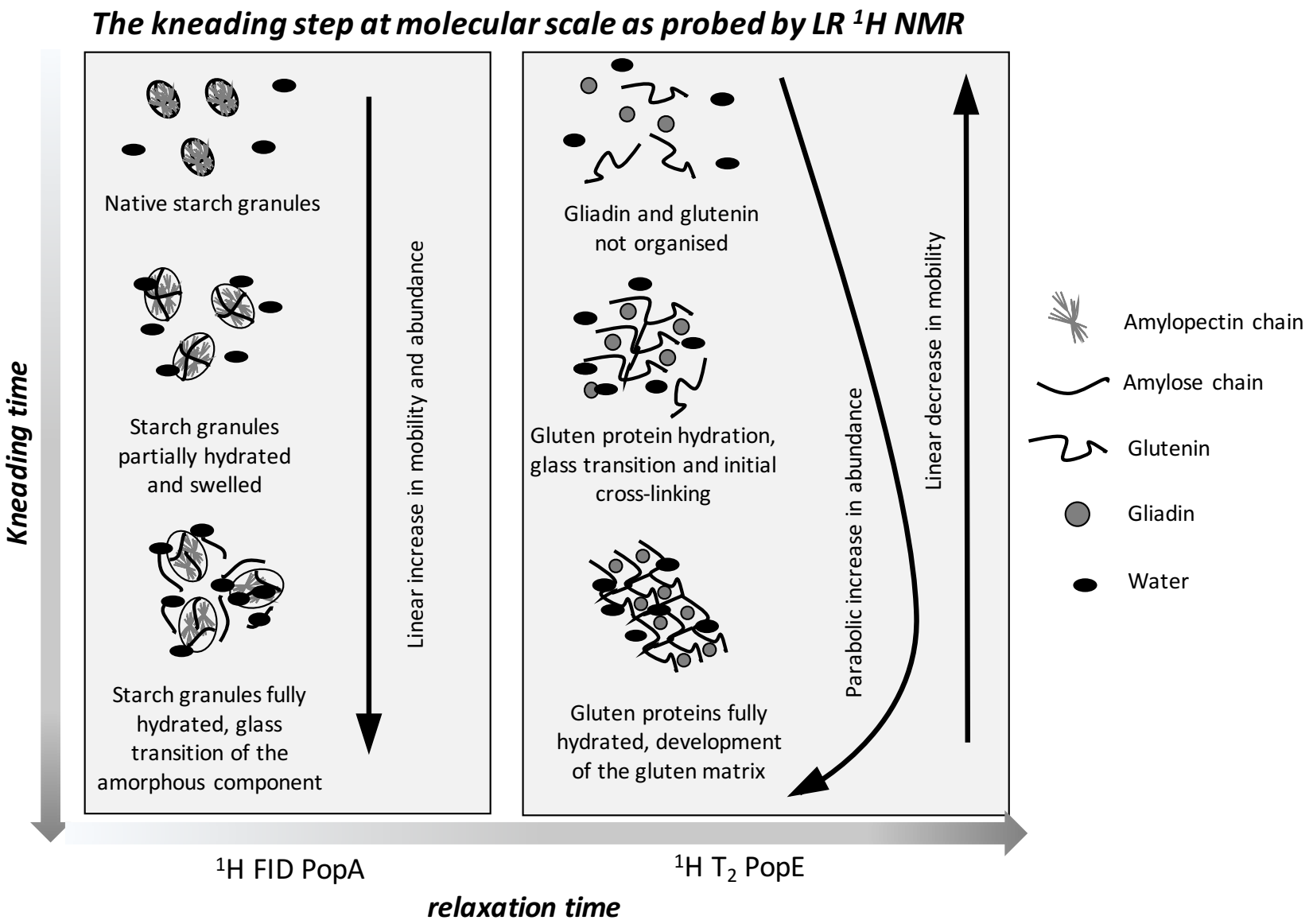

Figure 5 Schematic representation of the authors' interpretation of the ^{1}H NMR results obtained during the dough kneading step.