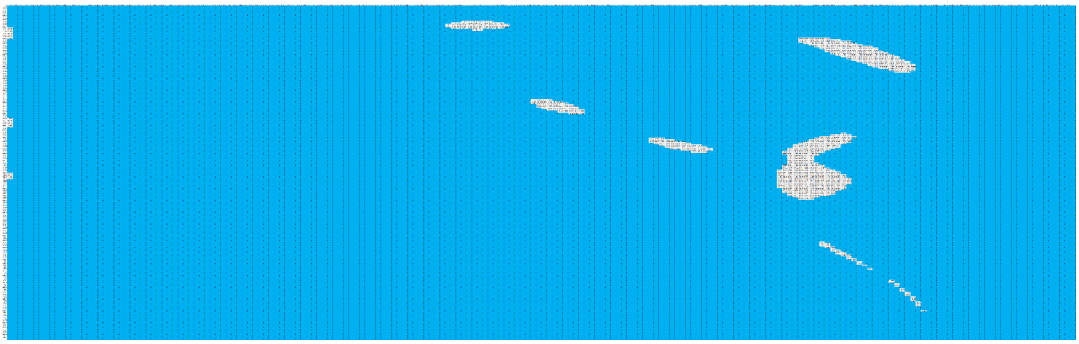
UNIVERSITA' DEGLI STUDI DI PARMA

DOTTORATO DI RICERCA IN SCIENZE DEGLI ALIMENTI

XXXI CICLO

EFFECT OF FORMULATION (WITH FOCUS ON GLUTEN FREE PRODUCTS) ON RHEOLOGICAL AND STRUCTURAL PROPERTIES OF PASTA AND BREAD



Coordinatore: Chiar.mo Prof. Furio Brighenti

Tutore: Chiar.ma Prof.ssa Elena Vittadini

Dottorando: **PAOLA LITTARDI**

Cover:

2nd place winner in the contest NMR WARS – NMR IS ART (2018)

PROTONS IN THE SKY (WITHOUT DIAMONDS)

Paola Littardi (E.Curti, E.Carini, E.Vittadini)

Università degli Studi di Parma – Department of Food and Drug

Gluten Free bread crumb at 7° day of shelf-life. Bi dimensional T_1_T_2Corr.
2017
20 MHz
A Friday evening, turning back home after a whole week of work on bi- dimensional excel T_1_T_2 maps, I was watching the blue sky. The clouds seemed like the protons populations that I was analysing all week long. Cool!!

UNIVERSITÀ DI PARMA

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CICLO XXXI

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Anni 2015/2018

Al mio bimbo, Emanuele

Ringraziamenti

Grazie mamma e papà perché, fino a qui, mi ci avete portata anche voi. Mi avete sostenuta sempre, in tutte le scelte, in tutte le decisioni, anche le più difficili. Grazie per avermi cresciuta come mi avete cresciuta e per avermi trasmesso tutti quei valori che vorrei tanto poter trasmettere anche io a mio figlio.

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Nucciaù, anche a distanza, sei stata in grado di darmi tanti buoni consigliù e di questo ti ringrazio infinitamente. Scusami per il tempo che ti ho rubatoùùù. Sei e resterai sempre il mio guruù per Bruker nonché mio "correlatore" per eccellenza. Leo, la calma che sei in grado di trasmettermi e le nostre "pause confessionale" mi sono state sempre di grande aiuto, soprattutto negli ultimi difficili mesi. (Rev. X_ECUR_ECAR)

Grazie anche a te Fatma per essere la mia “seconda mamma”, per essermi stata vicina e avermi tirato fuori dalle sabbie mobili quando non sapevo come fare. Ti ringrazio per le chiacchiere e le merende col the e i biscotti (quelli burrosi con i granelli di zucchero). Ti ringrazio per avermi aiutato a spremere le arance e tutta l’altra frutta.

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E comunque, adesso si, ti do il permesso, adesso si può davvero cominciare a festeggiare.

Beh siiii ☺ !!!

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Summary

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Food industry has, in recent years, tried to answer consumers' request for high quality gluten-free products, originating from a growing incidence of celiac and gluten intolerant individuals. Gluten free products quality is known to be challenged by the lack of the gluten network that confers unique rheological properties and has an important structural role in dough and final products. Even though considerable improvements were made in gluten-free products' quality, the research is still looking for suitable alternatives (ingredient and process) to compensate the absence of gluten.

To overcome this challenging situation, it becomes crucial to design new formulations, with traditional and/or innovative methods. To achieve such goals, there is intensive need to acquire deep understanding of the dynamics that determine the final quality of the product. In this perspective, this work aims to investigate how different gluten free formulations impacts on rheological and structural properties of the most popular cereal-based products, pasta and bread, as compared to gluten containing products (traditional recipe and formulated with non-usual ingredients).

The scientific approach followed within this PhD thesis is a "multidimensional approach", where macroscopic, mesoscopic, microscopic and molecular features of bread and pasta products made with different formulations (gluten containing and gluten free) were thoroughly discussed. Noteworthy, this work took also into

consideration: i) the different cooking phases in commercial formulations of pasta, ii) gluten free bread during storage, iii) the effectiveness of additives in retarding the gluten-free bread staling phenomenon. To enable a full assessment of the possible dynamics that govern the product's final quality, conventional as well innovative methods for characterization of product's physic chemical properties were used.

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Introduction

Introduction

This PhD thesis has focused on two of the most popular foods all over the word: pasta and bread. The quality of these products (in gluten free formulations) is markedly affected by the absence of the gluten network that has a key structural role in both products and significantly influences their quality. Therefore, this work was divided in two sections, the first focused on pasta and the second on bread.

PASTA

Traditional Italian dry pasta is one of the most popular food consumed in the world. This product is conventionally produced using two ingredients: wheat durum semolina and water (Malcolmson & Matsuo, 1993; Carini *et al.,* 2013a).

During the cooking process in boiling water, the two main components of semolina, starch and gluten, undergo relevant changes that are strictly connected to final product's quality. Starch gelatinization and polymerized gluten go through a competitive phenomenon toward water absorption during cooking (Petitot et *al.,* 2010) and with the progress of cooking, the starch fraction becomes soluble while gluten insoluble (Pagani *et al.,* 2007). Recently, novel ingredients have been used in pasta products, to meet health (nutritionally enriched pasta) or dietary (gluten-free pasta) requirements. The presence of such ingredients can strongly affect pasta processing and product quality in all its forms: raw, dry or cooked. It is well known that pasta formulation/process influence the quality characteristics of pasta and are strongly dependent on cooking time (Wood, 2009; Petitot *et al.* 2010; Bustos *et al.* 2011; Aravind *et al.,* 2012; Carini et *al.,* 2012; Lucisano *et al.* 2012; Islas-Rubio *et al.,* 2014; Bustos *et al.* 2015).

In gluten-free pasta, the lack of gluten network causes the leaching of starch fractions in the cooking water (increasing cooking loss), an increasing stickiness and an overall scarce acceptability.

The presence of additional ingredients, or the entire replacement of durum wheat semolina, can strongly affect pasta processing and its end-quality, as it was previously reported in a series of works (Fuad & Prabhasankar, 2010; Bustos *et al.,* 2015). Several studies have reported that inclusion of non-conventional ingredients in a formulation (vegetable, legumes, minor cereals and pseudo cereals) generally worsens physico-chemical properties (e.g. cooking and texture properties) of pasta, mainly due to the development of discontinuities in the gluten phase or the absence of gluten network (Tudorica *et al.,* 2002; Brennan & Tudorica, 2007; Petitot *et al.,* 2010; Mercier *et al.,* 2016). The effect of non-traditional ingredients on water dynamics during pasta cooking process in relation to rheological properties (Dinamic Mechanical Analysis) has been addressed in very few reports in the scientific literature (Del Nobile *et al.,* 2005; Chillo *et al.,* 2009).

Textural attributes (e.g. hardness and stickiness), degree of swelling, solid loss in cooking water, expansion ratio, water absorption index, colour, aroma and taste are the traditional parameters used for the determination of cooked pasta quality (Baiano *et al.* 2006; Chillo *et al.,* 2008a, b; Fuad & Prabhasankar, 2010; Benhur *et al.* 2015; Bustos *et al.,* 2015; Sereewat *et al.* 2015; Morreale *et al.,* in press). These properties are related not only to product's physico-chemical and textural properties, but also to molecular events occurring during cooking. In this optic, Magnetic Resonance Imaging, MRI (Bernin *et al.* 2014; Steglich *et al.* 2014), and Time Domain Nuclear Magnetic Resonance, TD-NMR, are able to provide information about molecular mobility and dynamics in complex foods (Van Duynhoven *et al.* 2010) such as noodle and pasta (Kojima *et al.* 2001; Carini *et al.* 2013b;

Carini *et al.* 2014; Curti *et al.* 2015a; Diantom *et al.* 2016). These techniques can be used to find the association between molecular dynamics, macroscopic and mesoscopic properties in different formulation of pasta.

BREAD

Gluten free bread is known to have poor quality in terms of quick hardening and shorter shelf-life as compared to gluten containing ones. This is due to the large amount of starch, that present a rapid onset staling and consistent amylopectin retrogradation (Gallagher *et al.,* 2004; Moore *et al.,* 2004; Osella *et al.,* 2005; Sciarini *et al.,* 2010; Arendt & Dal Bello, 2011; Cappa *et al.,* 2013; Ziobro *et al.,* 2013; Capriles and Arêas, 2014; Demirkesen *et al.,* 2014), and to the presence of more water molecules available for macroscopic migration and molecular redistribution among components (Gallagher *et al.,* 2004; Masure *et al.,* 2016). A variety of solutions has been proprosed such as the use different raw materials, the addition of different proteins, hydrocolloids, (Gallagher, 2009; Arendt & Dal Bello, 2011; Masure *et al.,* 2016).

Also, considering the large use of α-amylases with anti-staling action in traditional white bread (Hug-Iten *et al.*, 2003; Goesaert *et al.,* 2009a,b), and the impossibility to use it in starch reach products [due to its drastic debranching action on the amylose and amylopectin chains], it cannot be used in GF bread, where the gelatinized starch has an important structural role. Indeed, β-amylase, having a milder debranching action (Hug-Iten *et al.,* 2003), were considered as potential anti-staling agent in gluten free bread.

During storage different macroscopic, mesoscopic and molecular changes takes place in the product matrix, traditionally measured in terms of moisture content, activity water, texture, frozen water and retrograded amylopectin. A lot of difficulties in tracking these modifications in GF formulations are due to the variety and multitude of different

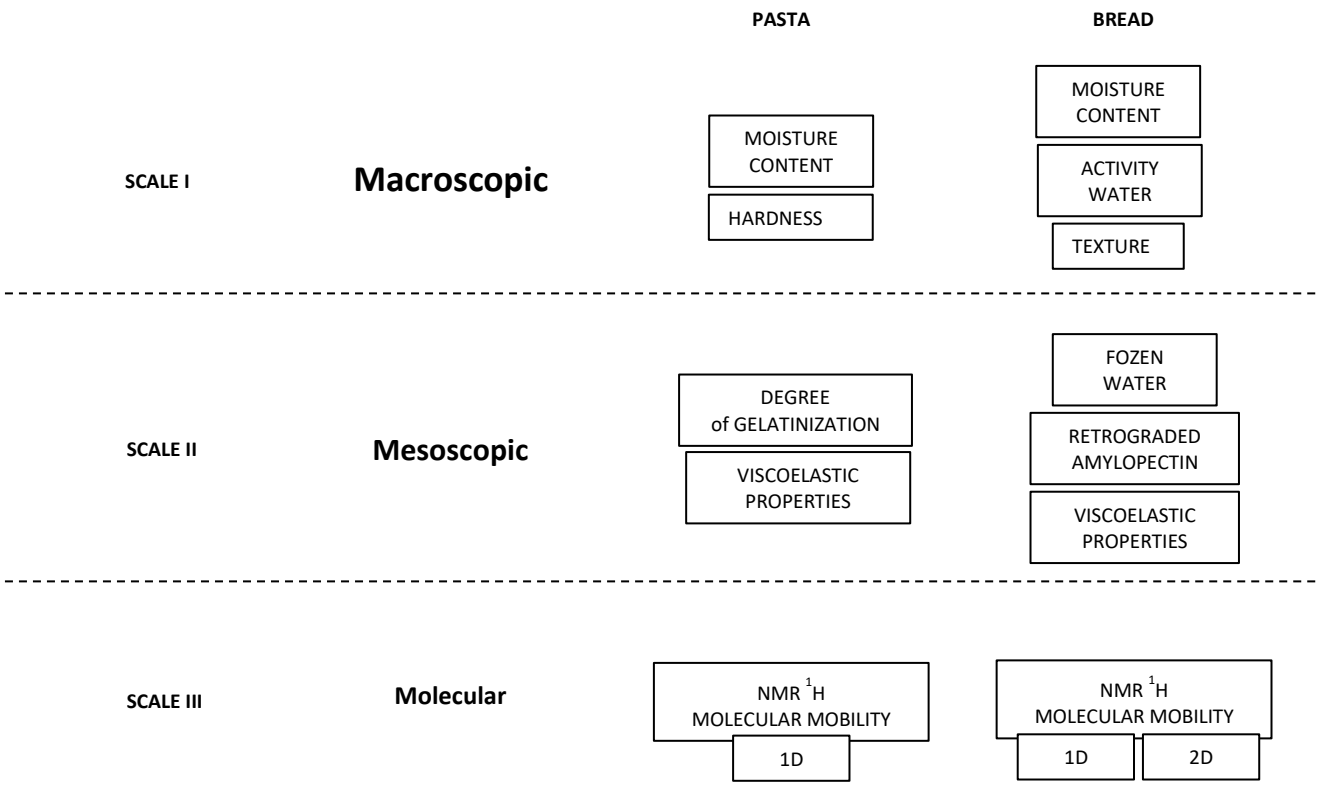
ingredient used to mimic the gluten network (Moore *et al.,* 2004; Ahlborn *et al.,* 2005; Zannini *et al.,* 2012). The bread staling is yet not well elucidated, and more information are necessary to understand the molecular dynamics of this phenomenon. From an analytical perspective, even though the use of Time Domain Nuclear Magnetic Resonance (TD-NMR) is well-documented in the investigation of molecular dynamics in regular bread during storage (Wang *et al.,* 2004; Sereno *et al.,* 2007; Curti *et al.,* 2011; Bosmans *et al.,* 2013; Curti *et al.,* 2015b; Curti *et al.,* 2017a;), this application is in its early stages concerning GF bread (Purhagen *et al.,* 2012; Hager *et al.,* 2014; Carini *et al.,* 2017), but it could be useful to understand the molecular dynamics in this product. Moreover, the use of two-dimensional (2D) NMR method (2D TD-NMR) have been recognized able to provide a better resolution of peaks than 1D TD-NMR (Curti *et al.,* 2017b).

Objectives

The workflow of this thesis is devised into two sections:

- section A. The entire cooking process, from uncooked to over cooked, of different formulations of commercial dry pasta was studied using a multidimensional approach with the aim to better understand the relevant features at macroscopic (moisture content and textural properties), mesoscopic (degree of gelatinization and viscoelastic behaviour) and molecular (proton molecular mobility) levels.
- section B. This part was dedicated to the characterization of the staling process in GF bread and the evaluation of the effect of beta-amylase as anti-staling agent in gluten-free (GF) bread. This part of work aims to apply 2D (T_1-T_2) NMR relaxometry, together with 1D NMR relaxometry and more traditional techniques, to investigate staling molecular dynamics in GF bread with different formulations, in an attempt to better highlight of molecular dynamics of these products. Moreover, also the study of β-amylase as potential anti-staling agent in GF bread was conduct, with the application of a multidimensional approach.

MULTIDIMENSIONAL APPROACH



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Section A: PASTA

A multi-scale characterization of the durum wheat pasta cooking process

A better elucidation of pasta cooking process

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Summary

A multi-level investigation was carried out to better characterize pasta from the uncooked (0 min) to the overcooked (14 min) product. Macroscopic, mesoscopic and molecular properties were measured and their changes were clearly related to the process of cooking: hardness and viscoelastic properties decreased (hardness from 98 to 8 N, tanδ from 0.33 to 0.20); moisture content and gelatinization degree increased; ^{1}H molecular mobility changed. Principal component analysis (PCA) explained 82% (PC1: 47.7% and PC2: 34.3%), enabling the differentiation by cluster of different cooking phases: uncooked pasta; undercooked pasta described by degree of gelatinization and rigid protons populations (FID, and T_2, and ^{1}H relative abundances); medium-cooked pasta described by higher mobility protons (T_2E1, T_2E2 and %PopE2); suggested cooking time described by viscoelastic properties and over-cooked pasta by molecular mobility parameters (T_2D, T_2E and %PopF). Nuclear magnetic resonance was a valuable tool to describe the entire cooking process, while viscoelastic properties effectively characterized suggested cooking time.

Keywords: pasta quality; cooking; ^{1}H NMR mobility; Dynamic Mechanical Analysis; Multivariate statistics, starch gelatinization.

Introduction

Pasta is a staple Italian food largely consumed worldwide and owes its popularity to its simple formulation (only two ingredients such as wheat durum semolina and water), its low cost, ease of storage, handling, and preparation, as well as to its versatility in meal preparation (Carini *et al.* 2013a). Quality of pasta is usually evaluated in terms of textural attributes (i.e. hardness, stickiness), degree of swelling, solid loss in cooking water, colour, aroma and taste after cooking in excess water (Baiano *et al.* 2006; Chillo *et al.* 2008a, b; Benhur *et al.* 2015; Sereewat *et al.* 2015). Cooked product's characteristics are closely related to pasta formulation/process and are strongly dependent on cooking time (Wood, 2009; Petitot *et al.* 2010; Bustos *et al.* 2011; Aravind et al., 2012; Islas-Rubio *et al.* 2014; Lucisano *et al.* 2012; Bustos *et al.* 2015).

In particular, textural characteristics of cooked pasta, as perceived by the consumers, are related to water dynamics and to the mesoscopic and molecular events occurring during cooking. Time Domain Nuclear Magnetic Resonance (TD-NMR) is able to provide information about molecular mobility and dynamics in complex foods (Van Duynhoven *et al.* 2010), and can be used in an attempt to relate molecular dynamics with macroscopic properties in a multi-level characterization approach of food items. With regards to pasta, TD-NMR has already been applied on noodles, shelf-stable pasta (Kojima *et al.* 2001; Carini *et al.* 2014; Curti *et al.* 2015; Diantom *et al.* 2016), and complex ready-to-eat pasta meals (Carini *et al.* 2013b), where molecular mobility importantly contributed to the understanding of textural attributes of the product. More advanced NMR techniques (e.g. Real-time Magnetic Resonance Imaging), in combination with microscopic investigation, has been used to study the cooking process of a single stand of spaghetti in glass tubes, to

mimic the traditional cooking process (Bernin *et al.* 2014; Steglich *et al.* 2014), without assessing textural and rheological properties.

In this work, the cooking process of pasta (from uncooked to overcooked product) was studied to better describe pasta features at different levels of investigation: macroscopic (moisture content and textural properties), mesoscopic (degree of gelatinization and viscoelastic behaviour) and molecular (proton molecular mobility). In particular, two unconventional techniques for investigating pasta properties were chosen [Dynamic Mechanical Analysis (DMA) and TD-NMR], to better unravel the changes occurring in viscoelastic and molecular properties during cooking. The effectiveness of the multiscale approach in describing the pasta cooking was assessed by multivariate statistics.

Materials and methods

Sample, cooking procedure and timing of analyses

Commercial pasta, ridged penne shaped, was purchased from a local supermarket. Pasta was made exclusively with durum wheat semolina, and its suggested cooking time (SCT) was 10 min, as indicated by the manufacturer. Pasta was cooked for different cooking times (1, 2, 5, 6, 8, 9, 10, 12, and 14 min) in boiling water (pasta/water ratio 1:10), drained, cooled and stored in a closed container (25°C, 15 min) to avoid water evaporation before analysis. Analyses were carried out and completed within 2 hours from the end of cooking [except for Differential Scanning Calorimetry (DSC) analysis, that required preliminary preparation]. The uncooked product (0 min) was also analysed. Three batches were analysed for each cooking time.

Macroscopic properties

Moisture content (MC, g of water/100 g of sample) was measured by weight loss by drying in a forced-air oven (ISCO NSV 9035, ISCO, Milan, Italy) at 105 °C to constant weight. At least three pasta pieces for each cooking time for each batch were analysed.

Pasta hardness was measured with a cutting test (flat blade, HDP/BS, 3 mm thickness, TA.TX2 Texture Analyzer, 5-kg load cell; Stable Micro Systems, Godalming UK). A single piece of pasta (single penna) was placed perpendicularly to the blade and completely cut (distance 30 mm; cut speed 2 mm/s; trigger force of 0.1 N). Force at break was taken as hardness (N). For each cooking trial (n=3), at least 7 "penna" samples were cut for each cooking time. All obtained values were used in the calculation of hardness mean..

Mesoscopic properties

The degree of starch gelatinization (DG%) was measured using a differential scanning calorimeter (DSC Q100, TA Instruments, New Castle, DE, USA) calibrated with indium (T = 156.6°C; H = 28.71 J/g) and mercury (T = −38.83°C, H = 11.40 J/g) following the procedure of Cleary & Brennan (2006). All samples were freeze-dried (Liophilizer LIO 5PDGT, 5Pascal, Milan, Italy), ground (Osterizer, Sunbeam, USA) and sieved (Giuliani Tecnologie, Torino, Italy) to collect particles <500 μm. Freeze-dried samples (60-70 mg) were mixed with 200 μL of distilled water, and aliquots of the suspension (60-70 mg) were placed into hermetic stainless-steel pans (Perkin Elmer, USA), that were heated from 25 to 120°C at 5°C/min using an empty pan as reference. After analysis, pans were pierced and dried at 105°C to constant weight to evaporate water and obtain the dry sample weight. Gelatinization peak enthalpy (J/g product) was normalized to the dry weight (J/g dry

sample). The degree of gelatinization (DG%) was calculated according to the following equation:

$$degree\ of\ gelatinization\ (DG\%) = \left[100 - \left(\frac{J/g\ cooked\ dried\ sample}{J/g\ uncooked\ dried\ sample} * 100\right)\right] \quad (1)$$

At least triplicate samples of pasta were analysed for each cooking time for each batch. Viscoelastic properties (storage modulus, E'; loss modulus, E''; phase angle, tan δ) were measured using a dynamic mechanical analyser (DMA – Q800, TA Instruments, New Castle, DE, USA), by developing a tailored method for pasta. A small pasta cylinder (3.85 mm diameter and 1.6-2.1 mm thickness) was extracted from each penna and analysed in compression mode (15 mm diameter parallel plate compression clamp) with a frequency sweep test (25°C). Amplitude was set to 15 μm, within the linear viscoelastic region (LVR) (determined with a preliminary strain sweep test). At least 9 penne were analysed for each sample for each batch.

Proton molecular mobility (Time-Domain NMR, TD-NMR)

^{1}H molecular mobility was measured with a low resolution (20 MHz) ^{1}H NMR spectrometer (the miniSpec, Bruker Biospin, Milano, Italy) operating at 25.0 ± 0.1°C. Cooked pasta samples were placed in a 10 mm NMR tube sealed with Parafilm® to avoid moisture loss during the experiment. Three NMR tubes were analysed for each cooking time for each batch.

^{1}H FIDs were acquired using a single 90° pulse [dwell time of 7 μs, recycle delay 1 s (>5T_1), 0.5 ms acquisition window, 900 data points] and fitted with two components

models, according to the following equations, where *y0* is the FID offset, *A* and *B* are the intensities of each relaxation component, T_A and T_B the apparent relaxation times:

$$F(t): y0 + A * exp\left(-{}^{t}/_{T_A}\right) + B * sin(c * t)/(c * t) * exp\left[-\left({}^{t}/_{T_B}\right)^2 * 0.5\right]$$

(2)

(for uncooked samples, 0 min; exponential and Gaussian, modified with a Pake function; Derbyshire *et al.* 2004; MATLAB 2016a, The MathWorks Inc. USA)

$$F(t): y0 + A * exp(-t / T_A) + B * exp[-(t / T_B)^2] \quad (3)$$

(for samples cooked at times ≥1 min; exponential and Gaussian; Le Grand *et al.* 2007; Sigmaplot, v6, Systat Software Inc. USA).

^{1}H T_2 relaxation time was measured with a Carr, Purcell, Meiboom and Gill pulse sequence (CPMG) [recycle delay: 1 s (≥5T_1); interpulse spacing: 40 μs; 8000 data points]. T_2 curves were analysed as quasi-continuous distributions of relaxation times (UPENWin, Alma Mater Studiorum, Bologna, Italy). Default values for all UPEN parameters were used. ^{1}H T_2 curves were fitted with a discrete exponential model (Sigmaplot, v.10, Systat Software Inc., USA) to obtain relaxation times and proton populations abundances.

Statistical analysis

Analysis of variance (ANOVA) was performed and significant differences among the mean values were calculated using Duncan's test ($p \leq 0.05$). Correlation coefficients (r) were computed using Pearson's coefficient ($p \leq 0.05$). Principal Component Analysis (PCA) was also performed based on the correlation matrix. PCA was firstly applied using all the dataset. Subsequently, the relevant features were discriminated based on the load scores (> ±0.6). The biplot generated by the relevant features was used to project cooking phases

into a plane of the first and second principal component. All analyses were performed using SPSS software (version 25.0, SPSS Inc., Chicago, IL, USA).

Results and discussion

Macroscopic properties

Moisture content (MC, % g water/100 g sample) of uncooked sample was 5.4% and significantly increased with increasing cooking time [e.g. from 53.8% (10 min) to 60.4% (14 min)], as consequence of water uptake (Fig. S1).

Pasta hardness was not measurable in the uncooked product, as pasta crumbled during cutting. Hardness went through three phases during cooking: a significant decrease from 1 (98.3 N) to 8 min (11.3 N), a plateau from 8 to 10 min (9.6 N) and a not significant decrease from 10 to 14 min (7.8 N) (Fig. S1). This progressive hardness decrease follows water absorption into the pasta matrix from the outer layers towards the inner core, with consequent starch gelatinization and progressive structural softening (Bustos *et al.* 2015).

Mesoscopic properties

DSC thermograms (not shown) of freeze-dried uncooked and cooked pastas were characterized by the presence of one endothermic transition in the range ≈55-85°C, peaking at ≈60-76°C, related to starch gelatinization of crystalline domains (Delcour & Hoseney, 2010). DG was 35.0% in samples cooked for 1 min, and progressively and significantly increased with increasing cooking time (Fig. 1). DG gradually increased to 50.7% at 9 min, suddenly increased to 81.1% at suggested cooking time (SCT, 10 min), and then gradually increased again to 92.4% after 14 min, indicating almost complete starch gelatinization

(Fig. 1). As expected, DG was highly correlated to MC (r=0.914; $p \leq 0.01$), confirming that starch gelatinization followed water uptake during cooking.

Pasta viscoelastic properties were measurable for cooking times longer than 5 min, as at shorter times cooked samples could not be properly loaded in the DMA probe, due to their fragility. Representative curves of E' and E'' versus frequency are reported in Fig. S2 showing a predominant elastic behaviour of pasta during the entire cooking process (E' always higher than E''). E', E" and tanδ values at a single frequency (25 Hz) are shown in Fig. 2. E' and E" decreased with increasing cooking time, indicating a weakening of the pasta matrix following the progressive water uptake, gluten plasticization/coagulation and starch gelatinization (Chillo *et al.* 2009). Tanδ was lower than 1 (Fig. 2), and steadily decreased with increasing cooking time. In contrast, Chillo *et al.* (2009) reported maximum tanδ at OCT (determined also by sensory analysis) in spaghetti analysed in a tensile mode. No significant correlations were found between E', E'' and any other parameter, while tanδ was significantly correlated to MC (r=0.708; $p \leq 0.05$). The lack of relation among mechanical properties measured at different levels of investigation (visco-elasticity by DMA and hardness by texture analyser), possibly suggested that these techniques provided different information of pasta matrix properties.

According to macroscopic and mesoscopic characterization, the significant increase of DG from 8 min of cooking did not correspond to relevant changes in hardness, possibly indicating that macroscopic textural attributes of pasta were less related to starch gelatinization and more associated to gluten coagulation and plasticisation. At a mesoscopic level, pasta viscoelastic behaviour changed more gradually during cooking, possibly indicating a mediated contribution of gluten and starch phases.

Molecular properties

^{1}H FIDs in uncooked pasta (0 min) had the fastest decay and the line-shape indicates predominance of crystalline structures (Derbyshire *et al.* 2004). ^{1}H FID protons are representative of the fastest relaxing components of the system and were previously attributed to solid CH protons of crystalline and amorphous starch not in contact with water (Kim & Cornillon, 2001; Bosmans *et al.* 2013; Curti *et al.* 2015). FIDs line-shape became progressively less steep over cooking, suggesting an increasing mobility of the pasta matrix, following water uptake and heating and resulting in multiple phenomena, including starch gelatinization and protein hydration and coagulation (Fig. S3).

^{1}H FIDs were fitted with a two-components model [equation (2) for uncooked sample and equation (3) for cooked samples], to obtain relaxation time (FID TA) and population A abundance (%FID A) (Fig. 3). Population B was not considered in the discussion, as protons relaxing at similar relaxation times were also observed in ^{1}H T_2.

FID TA significantly increased (Fig. 3) during cooking from ~17 μs (0 min) to ~31 μs (14 min) while its abundance (%FID A) significantly decreased from 83.8% (0 min) to 34.2% (14 min). These changes were most likely due to an increased mobility following water uptake and starch gelatinization during cooking, as confirmed by the highly significant correlations between both FID TA and %FID A with MC (r=0.848, $p \leq 0.01$ and r=-0.936, $p \leq 0.01$, respectively) and DG (r=0.952, $p \leq 0.01$ and r=-0.940, $p \leq 0.01$).

Representative ^{1}H T_2 distributions of pasta during cooking (Fig. 4) showed the presence of multiple ^{1}H populations (C, D, E, E1, E2 and F). Populations varied depending on cooking time and, according to the distributions line-shape, a discrete fitting was performed to obtain relaxation times (T_2C, T_2D, T_2E, T_2E1, T_2E2, T_2F) and relative proton abundances (%PopC, %PopD, %PopE, %PopE1, %PopE2, %PopF) (data not shown).

In uncooked samples, two ^{1}H populations were observed, the predominant population E (the more rigid, relaxing at ~0.5 ms, 92.6% of total protons) and F (the more mobile, relaxing at ~100 ms). During cooking, considerable changes were observed in ^{1}H T_2 distributions. Two additional populations (C and D) were found from 1 min cooking, relaxing at 0.16 and 1.70 ms and amounting to 17.1% and 14.5%, respectively. Populations C and D probably arose from the more rigid protons (relaxing at shorter times than the first experimental point of T_2 distribution) and they became progressively more mobile, with a relevant increase after SCT (10 min), reaching ~0.8 and ~6.1 ms (T_2C and T_2D, respectively) at 14 min. Populations C and D became less represented upon cooking, encompassing ~6.1% and ~9.3% at 14 min, respectively. T_2C was highly correlated to DG (r= 0.703, $p \leq 0.05$) and FID TA (r=0.729, $p \leq 0.05$), while T_2D showed high correlation with MC (r=0.758, $p \leq 0.05$), DG (r=0.891, $p \leq 0.05$), FID TA (r=0.908, $p \leq 0.05$) and %PopA (r=-0.893, $p \leq 0.01$). Population (F), attributed to lipids (Hemdane *et al.* 2017), did not show relevant changes over cooking. Protons belonging to populations C and D, could be tentatively associated to mobility changes related to amorphous starch gelatinization.

Population E showed the most marked changes during cooking. A single population E was visible at 0 (uncooked), 12 and 14 min, while two overlapped proton populations (E1 and E2), shifting to higher relaxation times with increasing cooking, were observed from 1 to 10 min. Population E became narrower above SCT, indicating a faster exchange and a more homogenous proton molecular mobility. T_2E1 and T_2E2 increased from 5.7 ms (1 min) to 18.2 ms (10 min) and from 13.2 ms (1 min) to 34.8 ms (10 min), respectively. PopE1 decreased from 45.9% (1 min) to 21.1% (10 min) and PopE2 consequently increased from 21.1% (1 min) to 62.1% (10 min).

As a result of the correlations between molecular, mesoscopic and macroscopic properties [T_2E and DG (r=0.708; $p \leq 0.05$), %PopE1 and hardness (r=0.675; $p \leq 0.05$), %PopE2 and hardness (r=0.675; $p \leq 0.05$)], it could be speculated that the mobility changes observed in E, E1 and E2 were related to molecular mobility of starch and gluten domains, as they underwent physico-chemical modifications over cooking (gluten plasticization and coagulation, starch gelatinization with the development of an amorphous matrix and crystalline domains loss). The unique Population E at 0 min could be attributed to ungelatinized starch. At 1 min, the presence of overlapping populations may be indicative of a heterogeneous molecular structure and a "hindered" and inhomogeneous protons exchange, as water penetrates into pasta and starch/gluten rearranged over cooking, exhibiting different degrees of mobility. From 2 to 10 min, the presence of two overlapped populations (E1 and E2) may be reflecting an enhanced exchange of protons in starch and gluten domains. When cooking was completed (12-14 min), starch was completely gelatinized and represented by Population E only.

Multivariate statistics (PCA)

Principal Component Analysis (PCA) was performed to give a global understanding of macroscopic, mesoscopic and molecular pasta properties changes during cooking (Fig. 5a). All the variables were considered except for hardness, %PopC and %PopD (score value $< \pm$ 0.6). The first two components (PC1 and PC2) explained 82% of variance (47.7% and 34.3%, respectively). PC1 was explained by T_2E, T_2F, T_2D, %PopF, %PopE1, T_2E2, %PopE, FID TA, T_2E1, %PopE2, T_2C, while PC2 was explained by Tanδ, MC, E', E'', %PopA and DG.

The projection of cooking times on the factorial space created by PC1 and PC2 (Fig. 5b) enabled the discrimination of the cooking process:

- Uncooked pasta was clearly separated from the cooked samples (no variables able to describe this stage);
- Undercooked pasta (1-2 min) was characterized by the highest %PopA and the lowest DG, FIDTA and T_2C;
- Medium-cooked pasta (5, 6, 8 and 9 min) was characterized by the highest values of T_2E1, T_2E2 and %PopE2 and the lowest %PopE.
- SCT (10 min) was characterized by the highest viscoelastic properties (E', E' and tanδ).
- Over-cooked pasta (12-14 min) showed the highest values of T_2D, T_2E and %PopF.

The cooking process evolved differently, at the considered levels of investigation, with different magnitudes of changes, in accordance to the complex phenomena that take place during cooking. PCA suggested that macroscopic parameters, moisture content and especially hardness, despite being expected to be more informative, were only able to partially describe pasta characteristics during cooking with the observation of an important contribution from the parameters at meso- and molecular levels of investigation (molecular mobility parameters and viscoelastic properties).

Conclusions

A multiscale approach was used to describe the pasta cooking process in terms of macroscopic, mesoscopic and molecular properties. Multivariate statistics allowed to identify different stages of cooking, that were associated to parameters characterizing different levels of investigation. Molecular mobility indicators (FID TA, %popA and T_2C)

and DG were able to describe undercooked pasta (1 and 2 min); medium-cooked pasta (5, 6, 8 and 9 min) was characterized by higher mobility protons (T_2E1, T_2E2, %PopE and %PopE2); suggested cooking time (10 min) was related to viscoelastic properties changes; over-cooked pasta (12 and 14 min) was described by other molecular mobility parameters (T_2D, T_2E and %PopF).

Molecular properties were the most descriptive parameters of pasta cooking, making TD-NMR a valid technique to describe the entire cooking process, while DMA and viscoelastic properties were more explanatory for cooked pasta at SCT.

In this perspective, the use of the same approach (with the further possibility to correlate these results with specific descriptive sensorial attributes) is envisaged to better understand pasta cooking process in other formulations, e.g. gluten free/high fiber pastas and to assess if the same parameters (or some of them) are able to describe the cooking process in the same way.

Conflict of Interest: The authors declare no conflict of interest.

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Legend to Figures

Fig. 1. DG during pasta cooking; different superscript letters indicate significant differences ($p \leq 0.05$).

Fig. 2. Viscoelastic properties of pasta E' (●), E'' (▲) and Tan δ (■) at 25 Hz during cooking.

Fig. 3. FID relaxation time (TA) (●) and population A abundance (%FID A) (▲) of pasta during cooking.

Fig. 4. Representative ^{1}H T_2 quasi-continuous distributions of pasta during cooking.

Fig. 5. PCA (a) and Projection of pasta cooking times (min) (b) on the factorial space created by PC1 and PC2.

Supplementary Files

Fig. S1. MC (●) and hardness (▲) during pasta cooking; different superscript letters indicate significant differences ($p \leq 0.05$).

Fig. S2. Storage modulus E' (solid black line), and loss modulus E'' (dashed grey line) at suggested cooking time, vs frequency.

Fig. S3. Representative ^{1}H FID pasta curves during cooking.

Figure 1.

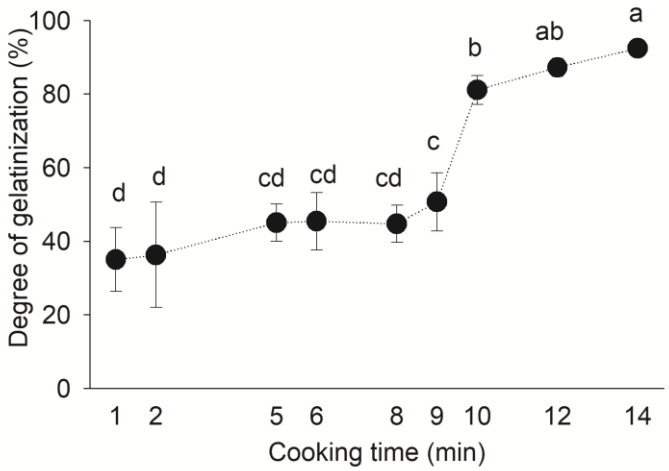

Degree of gelatinization (%)
100
80
60
40
20
0
d
d
cd
cd
cd
c
b
ab
a
1
2
5
6
8
9
10
12
14
Cooking time (min)

Figure 2.

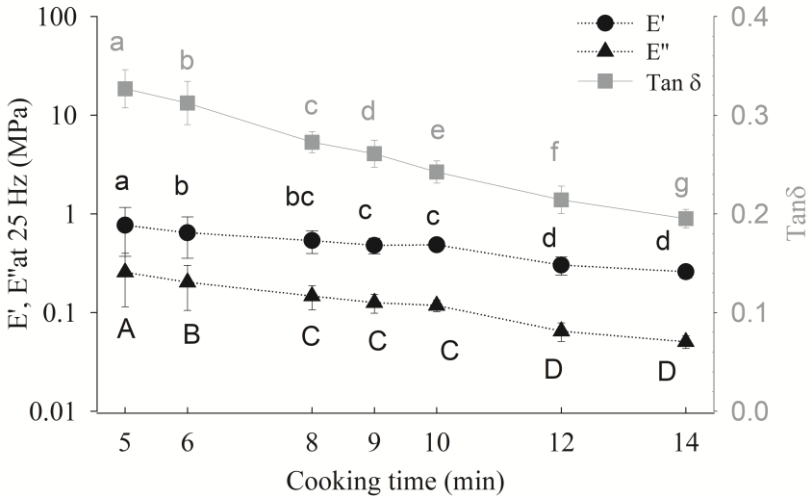

E'
E''
Tan δ
E', E''at 25 Hz (MPa)
Tanδ
Cooking time (min)
100
10
1
0.1
0.01
0.4
0.3
0.2
0.1
0.0
5
6
8
9
10
12
14
a
b
c
d
e
f
g
a
b
bc
c
c
d
d
A
B
C
C
C
D
D

Figure 3.

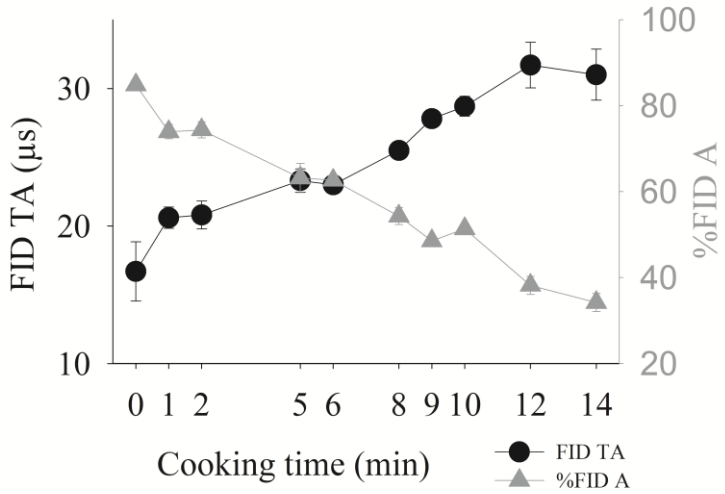

FID TA (µs)
30
20
10
100
80
60
40
20
%FID A
0 1 2 5 6 8 9 10 12 14
Cooking time (min)
FID TA
%FID A

Figure 4.

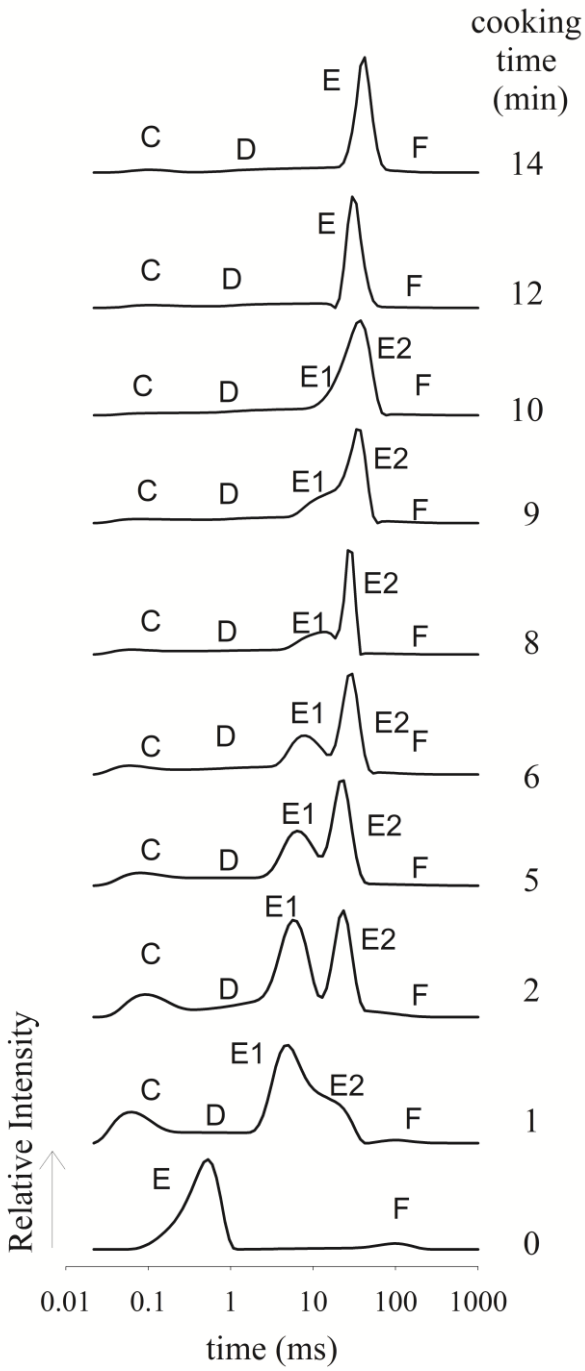

cooking time (min)
14
12
10
9
8
6
5
2
1
0
C
D
E
E1
E2
F
Relative Intensity
0.01
0.1
1
10
100
1000
time (ms)

Figure 5.

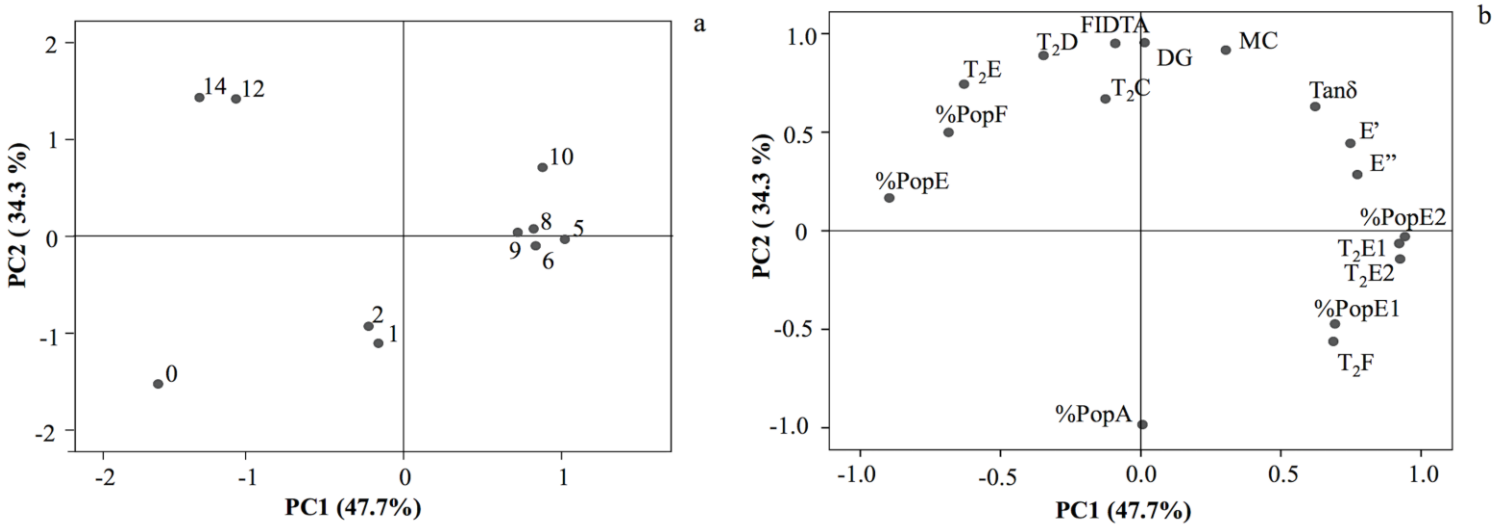

a
PC2 (34.3 %)
PC1 (47.7%)
14
12
10
8
5
9
6
2
1
0
b
PC2 (34.3 %)
PC1 (47.7%)
FIDTA
T2D
DG
MC
T2E
T2C
Tanδ
%PopF
E'
E"
%PopE
%PopE2
T2E1
T2E2
%PopE1
T2F
%PopA

Figure S1.

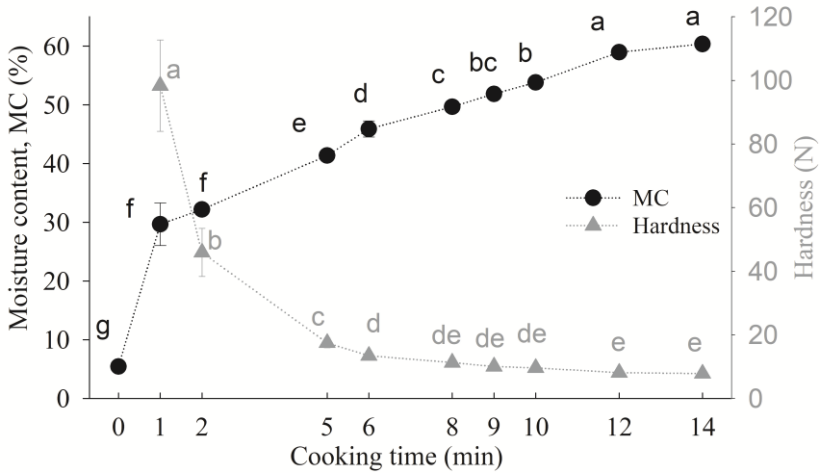

Moisture content, MC (%)
Hardness (N)
Cooking time (min)
MC
Hardness
0
10
20
30
40
50
60
0
20
40
60
80
100
120
0 1 2 5 6 8 9 10 12 14
g
f
f
e
d
c
bc
b
a
a
a
b
c
d
de
de
de
e
e

Figure S2.

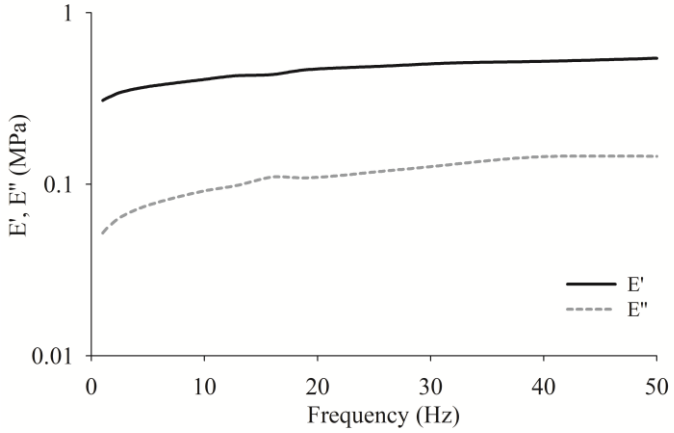
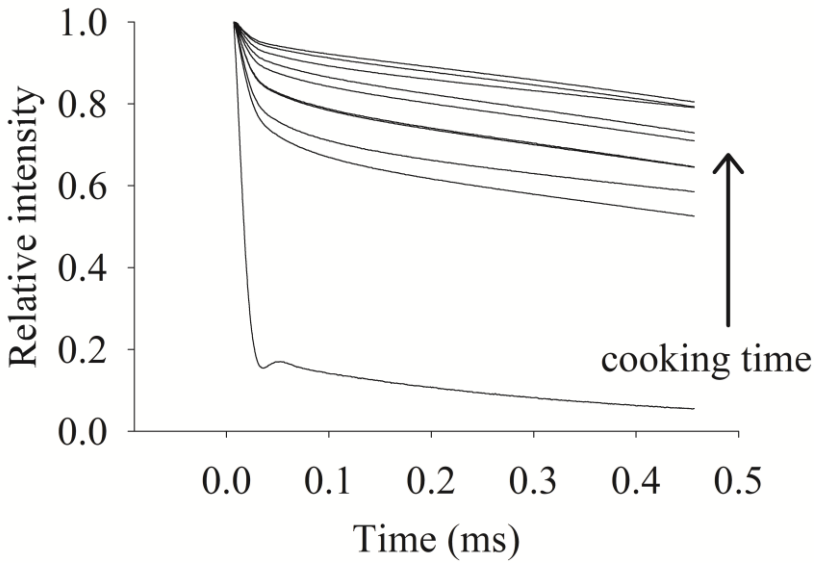


Figure S3.


1.0
0.8
0.6
0.4
0.2
0.0
Relative intensity
0.0
0.1
0.2
0.3
0.4
0.5
Time (ms)
cooking time

Littardi et al., to be submitted

Identification of typifying features of commercial pasta with different formulation during cooking with a multiscale investigation and discrimination analysis

Abstract

In this work quality changes in pasta with different formulations during cooking were investigated and the features better describing pasta were identified. For this purpose, whole grain (W), veggie (V) and gluten free (GF) pastas (from raw to overcooked) were analysed using a multiscale approach and discrimination analysis. Throughout 2-ways ANOVA emerged that the cooking time (CT) was the main factor influencing the studied properties overlapping the effect of pasta formulation PF), and consequently, each PT was considered singularly in discrimination analysis. Although principal component analysis (PCA) enabled the identification of some relevant features, a clear clustering of different cooking phases was not obtained. Nevertheless, partial least square (PLS) was effective in indicating viscoelastic properties and several NMR parameters as able to describe pasta behaviour during cooking and discriminate GF from gluten-containing pastas.

Keywords: pasta quality; formulation; cooking; ^{1}H NMR mobility; Dynamic Mechanical Analysis; Multivariate statistics; Partial Least Squares.

Introduction

Traditional dry pasta is produced with only two ingredients, wheat durum semolina and water (Malcolmson *et al*., 1993). However, ingredients with higher nutritional value (e.g. vegetable, legumes, minor cereals and pseudo cereals) can be use in specific pasta formulations to respond to the consumers' request for healthier products. Furthermore, due the increasing prevalence of people suffering from adverse reaction to wheat (e.g. celiac disease) (Marti and Pagani, 2013), the production of gluten-free pasta market has also gained much interest in pasta industry.

Unconventional ingredients and the use of components for the complete substitution of gluten, in gluten free pasta, can strongly affect pasta processing, and cooking quality in terms of texture properties, cooking loss, and firmness (Mercier *et al.,* 2016; Fuad & Prabhasankar, 2010; Bustos *et al.,* 2015). For instance, the inclusion of non-starch ingredients in fresh pasta formulation can affect dry matter, swelling index, protein content and textural attributes (Brennan & Tudorica, 2007). Soluble and insoluble fibre can also affect water uptake during cooking and weaken pasta structure (Tudorica *et al.,* 2002). The use of legumes flour reduced the optimal cooking time and water uptake, but increased cooking loss and hardness (Petitot *et al.,* 2010). Moreover, the presence of soy and carrot in fresh pasta altered quality attributes and water dynamics (i.e. water activity, frozen water content and proton molecular mobility), as reported by Carini *et al.,* 2012.

Traditionally, quality pasta attributes are evaluated at a macroscopic level in terms of color, solid loss in cooking water and textural properties such as hardness and stickiness (Mercier *et al.,* 2016), as they are the parameters that are more easily measured. Unconventional scientific techniques, such as dynamic mechanical analysis (DMA) and nuclear magnetic

resonance (NMR), have also been proposed to characterize pasta during the cooking process. Del Nobile *et al.,* (2005) investigated the effect of different protein levels of spaghetti pasta cooking on water dynamics, observing a lower rate of change of elastic modulus as a function of the cooking time at a higher protein content. Chillo *et al.,* (2009) studied the optimal cooking time of wholemeal semolina and maize-based spaghetti cooked using mechanical properties (tanδ, measured by dynamic mechanical analysis DMA). Low resolution ^{1}H Nuclear Magnetic Resonance, NMR (*Carini et al.,* 2014; Curti *et al.,* 2015), and Magnetic Resonance Imaging, MRI (Bernin *et al.,* 2014 and Steglich *et al.,* 2014) have been proven to be useful tools to describe water distribution and dynamics at a molecular level in pasta evaluating the effect of formulation. A multiscale approach coupled with statistical discrimination analysis was recently applied to investigate cooking of durum wheat conventional pasta, indicating that this approach is able to provide an interesting view of properties of pasta during cooking (Littardi *et al.,* submitted).

In this work, the same approach was applied to investigate cooking of commercial dry pasta with different formulations (whole wheat, veggie and gluten free), in terms of macroscopic, mesoscopic and molecular features. Relevant parameters in describing the quality changes for each type of pasta were identified by discrimination analysis.

Materials and methods

Sample and cooking procedure

Commercial whole wheat (W), veggie (V) and gluten free (GF) pasta (ridged penne) from the same producer were purchased from a local supermarket in the United States (Ithaca, New York). Pastas' list of ingredients were: W (durum whole wheat semolina, water); V

[wheat semolina, durum wheat flour, tomato puree, carrot puree (carrot, lemon juice concentrate, water), niacin, iron (ferrous sulfate), thiamine mononitrate, riboflavin, folic acid], GF [white corn flour (65%), yellow corn flour (29,5%), rice flour (5%), water, emulsifier: mono- and diglycerides of fatty acids]. The suggested cooking times (SCT) on the products labels were 9 minutes for W and GF, 10 minutes for V.

Pasta was cooked (1:10 pasta:water ratio) in boiling water for 1, 2, 5, 6, 8, 9, 10, 12, and 14 minutes. After draining, cooked pasta was stored at room temperature (25°C) for 15 min before analysis in a sealed vessel to avoid drying. The uncooked product (0 min cooking) was also analysed. For each pasta cooking time two batches were analysed (Littardi *et al.,* submitted).

Macroscopic assessment of pasta

Moisture content

Moisture content (MC %) was measured by drying at 105°C to constant weight with a forced air oven (ISCO NSV 9035, ISCO, Milan, Italy). For each cooking time, for each batch, triplicate analysis was carried out.

Texture

A TA.TX2 Texture Analyzer (Stable Micro Systems, Godalming, UK) equipped with flat blade (HDP/BS, 3 mm thickness, Stable Micro Systems, Godalming, UK) were used to measure pasta hardness (force at break, newton, N) with a cutting test (2 mm/s with and trigger force of 0.1 N). A single piece of pasta (penna) was placed perpendicularly to the blade and completely cut. The system was previously calibrated with a 5-kg load cell. At least 7 samples were analysed for each cooking time for each batch.

Mesoscopic assessment of pasta

Degree of gelatinization

All samples of pasta were lyophilized (Liophilizer LIO 5PDGT, 5Pascal, Milan, Italy) and ground with a blender (Osterizer, Sunbeam, USA) and passed through a 500 μm sieve (Giuliani Tecnologie, Torino, Italy). About 60–70 mg of the suspension (previously obtained mixing with a needle in an Eppendorf tube 60-70 mg of freeze-dried sample with 200 μL of distilled water), was placed into hermetic stainless-steel pans (Perkin Elmer, USA) using a micropipette. Following the method proposed by Cleary & Brennan (2006), the degree of starch gelatinization (DG%) was measured using a differential scanning calorimeter (DSC Q100, TA Instruments, New Castle, DE, USA). The device was previously calibrated with indium (T = 156.6°C; H = 28.71 J/g) and mercury (T = −38.83°C, H = 11.40 J/g). Samples were subjected to heating from 25 °C to 120°C at the speed of 5°C/min and using an empty pan as reference (Perkin Elmer, USA). Universal Analysis Software, (version 3.9A, TA Instruments, New Castle, DE) was used to obtain temperatures range (°C) and enthalpy (ΔH, J/g) of the gelatinization peak. At the end of analysis, pans were perforated to ease water evaporation during drying in a forced air oven at 105°C to constant weight (ISCO NSV 9035, ISCO, Milan, Italy). The gelatinization peak enthalpy (J/g product) was then normalized to the dried pasta sample weight (J/g dry sample). The degree of gelatinization (DG) at each cooking time was calculated according to the following equation:

$$degree\ of\ gelatinization\ \ (DG\%) = \left[100 - \left(\frac{J/g\ cooked\ dried\ sample}{J/g\ uncooked\ dried\ sample} * 100\right)\right]$$

Each cooking time for each batch was analysed at least in triplicate.

Viscoelastic properties

With the aim of studying viscoelastic properties [(storage modulus, E'; loss modulus, E''; and phase angle tan δ)] for penne shaped pasta, a tailored method was developed with the use of dynamic mechanical analyser (DMA – Q800, TA Instruments, New Castle, DE, USA).

A small cylinder of pasta (3.85 mm diameter, 1.6-2.1 mm thickness) was plucked out of each penna and treated with a 15 mm diameter parallel plate compression clamp applying a frequency sweep test (1-50Hz) in compression mode at the amplitude of 15 μm (preliminary determined with a strain sweep test to pinpoint the linear viscoelastic region, LVR). At least 9 penne were analysed for each sample for each batch.

Molecular assessment of pasta

^{1}H molecular mobility (Time Domain Proton Nuclear Magnetic Resonance, TD-NMR)

A low-resolution spectrometer (20 MHz, the miniSpec, Bruker Biospin, Milano, Italy) was used to evaluate proton molecular mobility, in terms of free induction decay (FID) and ^{1}H T_2 relaxation time at 25.0 ± 0.1°C. To avoid evaporation of water from the sample during the NMR test, specimen tube (10 mm internal diameter) containing pasta samples was sealed with Parafilm®. Two NMR tubes were analysed for each cooking time for each batch.

^{1}H FIDs were acquired using a single 90° pulse, followed by a dwell time of 7 μs, a recycle delay of 1 s (>5T_1), a 0.5 ms acquisition window and 900 data points. FIDs were fitted

with two components models (MATLAB 2016a; Sigmaplot, v6, Systat Software Inc. USA), according to the following equations, where *y0* is the FID decay offset, *A* and *B* are the intensities of each relaxation component, T_A and T_B the apparent relaxation times:

$$F(t): y0 + A * exp\left(-{}^{t}/_{T_A}\right) + B * \sin(c * t)/(c * t) * exp\left[-\left({}^{t}/_{T_B}\right)^2 * 0.5\right]$$

for raw samples corresponding to 0 minutes of cooking (exponential and Gaussian, modified with a Pake function; Derbyshire *et al.,* 2004, MATLAB 2016a, The MathWorks Inc. USA);

$$F\ (t): y0 + A * exp(-t\ /\ T_A\ ) + B * \ exp[-(t\ /\ T_B\ )^2]$$

for samples cooked at times longer than 1 min (exponential and Gaussian; Le Grand *et al.,* 2007; Sigmaplot, v10, Systat Software Inc. USA).

A Carr, Purcell, Meiboom and Gill pulse sequence (CPMG) (RD = 1 s, interpulse spacing = 40 μs, data points = 8000) was used to measure ^{1}H T_2 relaxation time. ^{1}H T_2 quasi-continuous distributions of relaxation times were obtained (UPENWin software Alma Mater Studiorum, Bologna, Italy) and were fitted with a discrete exponential model (Sigmaplot, v.6, Systat Software Inc., USA) to obtain relaxation times and proton populations abundances (%).

Statistical analysis

Analysis of variance (one-way ANOVA) was conducted to evaluate the effect of cooking time (CT) on each parameter for each pasta formulation (PF) at a significance level of $\alpha = 0.05$.

Significant differences among the mean values were calculated using Duncan's test ($p \leq 0.05$). Two-way ANOVA was performed taking in consideration two fixed factors (PT and CT). The partition of total variance of sum squares was computed to evaluate the contribution of single factors and their interaction in the variability of each parameter. Correlation coefficients (r) were computed using Pearson's coefficient ($p \leq 0.05$). To determine the effectiveness of the studied parameters in the description of cooked pasta quality, multivariate statistics, unsupervised (Principal Component Analysis, PCA) and supervised (Partial Least Squares, PLS). Both analyses were conducted on average values due to unequal number of determinations among properties.

PCA was performed based on correlation matrix. The relevant features were discriminated based on the load scores (>±0.6). As for PLS, venetian blinds cross-validation method was used for calibration. The prediction performance of the model was evaluated using coefficient of determination (R^2), root-mean-square error of calibration (RMSEC) and the root-mean-square error of prediction (RMSEP). The variable importance in the projection (VIP>1) was used to discriminate the relevant variables in the model.

All experimental data were analysed using SPSS (version 25.0, SPSS Inc., Chicago, IL, USA).

Results and discussion

Preliminary evaluation of the effect of cooking time and formulation on pasta properties

In the optic to evaluate the effect of cooking time and formulation on the studied quality features, the sum square percent of the studied factors was calculated based on the between-subjects' effects. 2 ways-ANOVA revealed that all the studied parameters were significantly influenced by both pasta formulation (PF) and cooking time (CT), as well as their interaction (PF*CT) (Table 1). Notably, the effect of CT was the most relevant (SS% between 65 and 98%) in the determination of the majority of the studied properties (MC, hardness, DG, Tanδ, FID TA, %FID A, T_2C, %PopC, T_2E, %PopE, T_2E1, %PopE, T_2E2, %PopE2). PF influenced T_2D, while PF*CT assumed importance in the determination of T_2F and %PopF. The contribution of both PF and PF*CT was relevant for E', E'', %PopD. Overall, CT was the more important controlling factor, that possibly partially hid the effect of PF. Therefore, the effect of CT on quality was studied separately for each type of pasta.

Effect of pasta formulations on cooking process

Macroscopic properties

Regardless of PF, increasing CT caused significant increase of MC following water uptake (Table 2). MC of uncooked product was ~6.1%, ~4.6%, ~7.6 % (g water / 100 g product) in W, V and GF respectively. After 1 min, a considerable increase in MC was observable in all samples (20% points in W and V, 27% in GF). At suggested cooking time (SCT), W

and GF (9 min) had ~52%, while V (10 min) had ~54%. All the overcooked samples (at 14 min) showed a moisture content ~59-60%.

Concerning texture, pasta fragility did not allow to measure hardness in the uncooked products (Table 2). Afterward, during the cooking process and independently of PF, hardness significantly decreased, as expected (Gonzalez *et al.,* 2000; Carini *et al.,* 2012; Diantom, *et al.,* 2016). Such a result is primarily due to water penetration and consequent starch gelatinization starting from the surface of the product and then proceeded toward the centre raising softness of the product (Cunin *et al.,* 1995; Bustos *et al.,* 2015). Noteworthy, after 1 min of cooking, GF was the hardest pasta (~90N, respectively) followed by V (~74N), while the softer was W (~46N) probably due to the presence of fibre. At SCT, hardness was ~10N in V, W and ~13N in GF. For overcooked pasta (at 14 min), hardness drastically decreased (about 7-8 N) for all samples.

Mesoscopic properties

Two endothermic DSC peaks (data not shown) were observed in W, V and GF in the DSC thermograms. The first peak at about 61-77°C was descriptive of starch gelatinization of crystalline domains, while the second peak at about 92-97°C was attributed to the fusion of lipid-amylose complexes (Zweifel *et al.,* 2000). The degree of gelatinization (DG) was calculated integrating the first DSC peak only. DG significantly increased during cooking in all samples (Table 2). V pasta followed a more gradual increase of DG during cooking, with 38% at 1 min and ~95% at SCT (10 min) and maintaining approximately this value also in overcooked samples. W started from 15% at 1 min, increased drastically at 10 min (~76%) and reach 90% in overcooked samples. GF started from ~56% (1 min), increased

substantially at SCT (~80%, 9 min) and reach ~82% in the overcooked sample. High correlations were observed between DG and MC in V (r=0.971; $p \leq 0.01$), W (r=0.929; $p \leq 0.01$) and GF (r=0.908; $p \leq 0.01$), being indicative of the strict connection between water uptake and starch gelatinisation regardless of formulation.

Storage modulus (E'), loss modulus (E'') and tanδ were measured with dynamic mechanical analysis (DMA). Only samples cooked for times longer than 5 min were analysable due to fragility and breaking of the shorter times cooked samples during preparation and analysis. E' curves (versus frequency) showed always higher values then E'' curves and E' never intersected E'' at all cooking times considered. Such a result highlighted a predominant elastic behaviour of the studied samples independently of formulation during the entire cooking process. Viscoelastic properties (E', E'' and Tanδ) were then compared at a single frequency of 25 Hz (Table 2) with the progress of cooking. E' decreased in W with increasing cooking time, while they increased until 9 min and then decreased in V and GF. This different behaviour could probably be ascribable to plasticization of proteins to the changes occurring in protein and starch (gelatinization) as a result of water uptake in relation to formulation. Remarkably, E'' followed the same trend of E' over cooking, and constantly decreased in W while increased until 9 min and then decreased in V and GF (Table 2). Tanδ steadily decreased (Table 2) with increasing cooking time independently of formulations (from ~0.30 to ~0.20 in V and W and from ~0.25 to ~0.16 in GF). This was in contrast with Chillo *et al*. (2009) who reported the maximum value of tanδ (in standard, whole meal, and maize based pastas) at the optimal cooking time. This discrepancy might be explained by the different pasta shape and experimental conditions (spaghetti and tensile mode, respectively). Tanδ was also

correlated with MC in all PF (r=0.749, $p \leq 0.05$; r=0.781, $p \leq 0.01$; r=0.718, $p \leq 0.01$; in V, W and GF, respectively), being indicative of the connection between viscoelastic properties and water uptake. Viscoelastic properties were also correlated with DG only in V and W (r=0.665, $p \leq 0.05$; r=0.713, $p \leq 0.05$ respectively), indicating that different and probably more complex dynamics in the starch phase in GF.

Molecular mobility

Solid CH protons of crystalline and amorphous starch not in contact with water were investigated with the evaluation of the most rigid protons population (^{1}H FID), presenting relaxation times shorter than 500 μs (Kim & Cornillon, 2001; Bosmans *et al.,* 2012; Curti *et al.,* 2015). The more mobile protons relaxing in the range 0.1 – 1000 ms were evaluated with ^{1}H T_2. FIDs of uncooked pasta showed a very fast initial decay and a hump in the second part. This shape was attributed to crystalline domains Derbyshire *et al.,* 2004; Van Duynhoven *et al.,* 2002) of starch, which in fact progressively changed with increasing CT in all pastas: the hump disappeared, and the line of proton decay decreased its slope (data not shown). These two events indicated increasing mobility following water uptake by macromolecules during the cooking process and starch gelatinization. The analysis of FIDs data was limited to protons belonging to population A, because population B included protons observed also in the ^{1}H T_2 time window.

The relaxation times (FID TA, ms) and the relative ^{1}H abundances (%PopA), obtained by bi-exponential model fitting of ^{1}H FIDs, are reported in Table 3.

Relaxation time FIDTA increased significantly during cooking: from ~ 17 μs at 0 min to ~ 31 μs in W and from ~10 μs to ~ 28 μs in V. GF was the less mobile sample, starting from

~ 9 µs and reaching ~ 21µs. %PopA significantly decreased in all pastas during cooking, following moisture content increase in consequence of water uptake (84-85% at 0 min and 35-40% at 14 min). These two events denoted the increase mobility of crystalline domains protons and starch gelatinization during cooking, as confirmed by high significant correlations of FIDTA and %PopA with both MC and DG ($r > \pm 0.800$, $p \leq 0.01$).

Representative ^1H T_2 distributions of pasta as function of cooking time (Figure 1) were indicative of multiple ^1H populations. A discrete fitting was performed to better characterize T_2 mobility, according to the line-shape of the distributions, indicating the presence of 6 protons populations in the majority of cases (Table 3).

In raw pasta samples ^1H T_2 distributions suggested the presence of two ^1H populations that were named population E (the more rigid relaxing in the range 0.1-1 ms) and F (the more mobile relaxing at about 100 ms). In uncooked GF population E suggested the presence of two overlapped populations E1 and E2. Population E (in W and V), or the sum of E1 and E2 (in GF) were predominant in all uncooked products representing ≥ 90% of total protons.

With the progress of cooking, severe changes were observed in ^1H T_2 distributions. W and V showed a similar evolution with the presence of two populations (C and D) at early stages of cooking (1-2 min), probably arising from more rigid protons (relaxing at shorter times than the first experimental point of T_2 distribution) that became more mobile with water uptake. Population C became evident at 1 min both in W and V, while D was observed at 1 min in W and 2 min in V. Moreover, population C in V at 1 min (relaxing at 0.83 ms) was represented by a broader peak (~40%), indicating a more inhomogeneous mobility of these protons, while it was less mobile (0.24 ms) and less represented (~20%) in W. Populations C and D in GF pasta at 1 min of cooking showed relative abundances of ~19 and ~13%,

respectively, and relaxed at 0.14 ms (T_2C) and 1.31 ms (T_2D). During cooking population C became progressively more mobile with some differences in the different pastas. In W the larger increase was at 8 min (T_2C~0.6 ms), in V at 12 min (T_2C~0.8 ms), while in GF in the overcooked product at 14 min (T_2C~0.7 ms). The relative abundance of population C decreased gradually to 4.6-7.4% in all types of pasta (14min). Also, population D became progressively more mobile and less represented over cooking following the same dynamics of population C. T_2D was ~3.0 ms (in W at 1 min and in V at 2 min), and ~1.3 ms in GF at 1 min and increased to 5.5-7.5 ms at 14 min in all pastas. T_2C and T_2D and its relative abundances seemed to be correlated ($r > \pm 0.6$, $p \leq 0.05$) with FIDTA and %PopA, indicating that these populations maybe derivate from more rigid protons that became more mobile due to water uptake. Moreover, %PopC and %PopD were correlated ($r > 0.7$, $p \leq 0.01$) with hardness tentatively indicating that the abundance of these two populations could be associated to the texture of pasta measured at macroscopic level.

Population E or its two "components", E1 and E2, showed different evolution during cooking in the different types of pasta (Figure 2). In uncooked, at early stages of cooking (until 2 min in W, until 5 min in V) and for cooking times longer than 12 min in W and V, population E was only observed and generally shifted to higher mobility with increasing cooking time, starting from 0.3-0.4 ms and becoming stable at about 25-30 ms at 12-14 min. In GF the predominant population was represented by the presence of E1 and E2 in the uncooked product and during the entire cooking process.

Discrete fitting data (Table 3) generally indicated a mobility increase during cooking in both populations E1 and E2 (in GF from ~0.3 to ~12 ms; in W and V from ~0.8 to ~27 ms, respectively, depending on pasta formulation), with relative abundance of population E1

decreasing from a maximum of 51% (at 1 min, in GF) to a minimum of 18% (at 12 min, in GF) and with a consequent increase in population E2, from a minimum value of 16% (at 2 min, in GF) to a maximum of 71% (at 12 min, in GF). Moreover, populations E1 and E2 became more clearly distinguishable and better resolved over cooking (from 1 to 8 min in GF), and then gradually merged into a single population E at 12 min. Different was the evolution of this two populations in W and V, in which E1 and E2 were never well resolved and defined.

The more mobile population (F) generally did not show relevant changes over cooking oscillating in the range ~50-140 ms and always with an abundance of about 1-10% and being attributable to protons of the lipid fraction.

The mobility changes observed in protons populations C, D, E, E1 and E2 indicated an interplay of the different proton domains in the considered pastas. Aside from the more relevant macromolecules modifications/water uptake and starch gelatinization (for all samples) and gluten plasticization and coagulation in gluten-containing samples, non-conventional ingredients could have also had an important role in modulating molecular mobility over cooking.

Identification of relevant features through discriminant analysis

In a first step, all the dataset was subjected to discrimination analysis (PCA and PLS), including durum wheat pasta, as a control (STD; Littardi *et al.,* submitted). The use of PCA did not allow for a defined clustering of the different stages of cooking on the basis of the studied parameters. PLS, based on supervised features, showed that the latent variables (LV1 + LV2) explained 49.25% of the variance (29.65% and 19.60% respectively LV1 and LV2),

due to the very different formulations of pastas considering all the parameters. The PLS graph (Figure 2) showed that only GF was clearly separated from the gluten-containing pasta samples (STD, W and V). These results suggested that the behaviour of pasta during cooking dynamics/kinetics was closely related to formulation.

Therefore, to identify the most relevant features in describing the cooking process, each PF was separately evaluated (Table 4). Using PCA, STD, V and GF were described as a function of MC, DG, viscoelastic properties (E', E'', Tanδ), NMR parameters (FID TA, %PopA, T_2C, T_2D, T_2E,T_2E1, T_2E2, T_2F, %PopE, %PopE1, %PopE2, %PopF), scoring values $< \pm 0.6$, while for W, T_2F and %PopF, were not considered. As expected, PLS was more discriminative, indeed in the case of STD, the most important variables were E', E'', FIDTA and T_2F; for V and W the more relevant variables were FID TA, T_2E1, T_2E2, T_2C, %PopD, %PopE, %PopE1 and %PopE2; and GF pasta showed a slightly different situation, with the most relevant variable being E', E'', FID TA, %PopE1 and %PopE2. These results underlined the importance of proton molecular mobility parameters (measured with TD-NMR) and viscoelastic properties (carried out with DMA) in describing the behaviour of different type of pasta during cooking.

Conclusions

In the light of the obtained results, multivariate statistic imparted that the effect of cooking time was the most relevant in the determination of the major part of the studied properties. Therefore, each pasta type was studied separately. Macroscopic, mesoscopic and molecular parameters were subjected to discriminative analysis that gave different results. Throughout PCA (based on unsupervised features), a high number of parameters, chiefly determined

using NMR, were able to describe each pasta behaviour during cooking process. However, no clear clustering of cooking phases was found. Using a PLS (based on supervised features), a smaller set of parameters of mesoscopic [DMA (E', E'')] and molecular parameters [NMR (FID TA, T_2C, %PopE, T_2E1, %PopE1, T_2E2, %PopE2, T_2F)] was efficient in discriminating GF pasta from those gluten containing. These findings underlined that integrating DMA and TD-NMR techniques can be a valid approach to describe the pasta cooking process.

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Table 1.

		MC	Hardness	DG	E'	E''	Tanδ	FID TA	%FID A	T_2C	%PopC
PF	SS%	0.54	6.53	5.31	43.96	40.23	18.06	22.29	1.46	12.19	8.46
	sig	***	***	***	***	***	***	***	***	***	***
CT	SS%	98.64	82.29	83.37	18.25	24.14	76.94	67.48	96.22	65.08	73.67
	sig	***	***	***	***	***	***	***	***	***	***
PF*CT	SS%	0.82	11.18	11.32	37.80	35.63	5.00	10.23	2.32	22.73	17.87
	sig	***	***	***	***	***	***	***	***	***	***

		T_2D	**%PopD**	**T_2E**	**%PopE**	**T_2E1**	**%PopE1**	**T_2E2**	**%PopE2**	**T_2F**	**%PopF**
PF	SS%	73.05	40.21	2.76	0.82	1.01	1.01	4.59	4.28	10.79	7.84
	sig	***	***	***	***	***	***	***	***	***	***
CT	SS%	11.21	17.24	96.65	86.26	93.15	93.15	85.88	93.54	36.54	51.23
	sig	***	***	***	***	***	***	***	***	***	***
PF*CT	SS%	15.73	42.55	0.59	12.92	5.85	5.85	9.53	2.18	52.67	40.93
	sig	***	***	***	***	***	***	***	***	***	***

Table 1. F significance level and sum square percent of the studied parameters and their interactions effects (***: $p \leq 0.001$; SS: sum of squares; CT: cooking time; PF: pasta formulation)

Table 2.

	MC (%)			Hardness (N)			DG (%)			E' (MPa)			E'' (MPa)			Tanδ		
CT (min)	W	V	GF	W	V	GF	W	V	GF	W	V	GF	W	V	GF	W	V	GF
0	6.12i	4.62h	7.63i															
1	27.06h	24.98g	34.31h	45.81a	73.8a	90.48a	15.39a	37.78a	55.88a									
2	34.92g	31.61f	38.23g	26.48b	59.77b	84.74b	26.1b	46.5b	40.62a									
5	45.72f	40.42e	46.57f	14.83c	18.19c	43.59c	43.55c	52.31bc	43.66a	0.376b	0.26de	0.152bc	0.112b	0.081b	0.039b	0.298a	0.31a	0.259a
6	46.08f	45.52d	45.4e	13.09c	15.56cd	26.15d	54.69d	56.37c	45.18a	0.515a	0.186ef	0.172bc	0.16a	0.053c	0.041b	0.31a	0.285b	0.235b
8	49.24e	45.56d	49.67d	9.9d	11.32de	15.19e	58.78d	73d	52.13a	0.197c	0.367bc	0.126bc	0.052c	0.095b	0.03bc	0.264b	0.261c	0.239b
9	51.62d	50.49c	51.83c	9.82d	9.68e	12.75f	53.55d	84.98e	80.4b	0.228c	0.74a	0.361a	0.057c	0.187a	0.079a	0.248bc	0.253cd	0.225b
10	52.95c	54.22b	56.76b	8.93de	10e	10.69f	76.55e	95.74f	77.01b	0.194c	0.173f	0.22b	0.045c	0.042c	0.044b	0.233c	0.243d	0.205c
12	56.09b	56.44ab	56.71b	7.84e	8.98e	7.95g	90.76f	90.6ef	84.85b	0.125d	0.418b	0.208b	0.029d	0.089b	0.039b	0.235c	0.212e	0.187d
14	58.82a	59.75a	59.63a	7.23e	8.07e	7.28g	89.77f	89.55ef	82.44b	0.136d	0.299cd	0.084c	0.028d	0.06c	0.014c	0.203d	0.2e	0.167e

Table 2. Moisture content (MC), hardness, degree of gelatinization (DG) and viscoelastic properties (E', E'' and Tanδ) for whole (W), veggie (V) and gluten free (GF) pasta during cooking. Different superscript letters indicate significant differences ($p \leq 0.05$).

	FID TA (ms)			T_2 C (ms)			T_2 D (ms)			T_2 E (ms)			T_2 E1 (ms)			T_2 E2 (ms)			T_2 F (ms)		
CT (min)	W	V	GF	W	V	GF	W	V	GF	W	V	GF	W	V	GF	W	V	GF	W	V	GF
0	0.017i	0.01h	0.009g							0.38e	0.26f				0.25g			0.83f	51.59f	76.45g	92.01c
1	0.022h	0.018g	0.019f	0.244c	0.832a	0.142c	3.01c		1.31e	8.4d	3.65e				4.93f			15.68e	125.88b	88.28f	112.29a
2	0.023g	0.022f	0.019f	0.261c	0.281c	0.163c	3.62b	3.09c	1.49e	9.32c	9.25d				5.68e			17.72d	79.93d	109.8cd	89.26cd
5	0.025f	0.024e	0.021de	0.177d	0.472b	0.181bc	2.05e	5.01b	1.76de		15.66c		7.59d		7.48d	20.44d		20.81c	135.88a	109.88cd	74.17d
6	0.025f	0.024e	0.02e	0.164d	0.231c	0.161c	1.34f	2.28d	1.77de				7.22d	7.71c	8.04d	19.37e	20.23d	20.71c	99.46c	138.34a	120.54a
8	0.027e	0.026d	0.021cd	0.612d	0.246c	0.183bc	5.8f	2.26d	2.04cd				8.19c	8.97b	9.59c	23.01c	24.33c	22.79b	51.53e	113.4bc	94.25bc
9	0.029d	0.027c	0.022bc	0.78c	0.251c	0.221b	7.9d	2.32d	2.23cd				11.17a	9.39b	10.81b	28.04a	22.78b	24.4a	114.47a	122.87b	103.94abc
10	0.03c	0.029a	0.023a	0.813d	0.302c	0.23b	7.41e	2.66cd	2.24cd				10.25b	11.31a	11.31ab	25.92b	27.21a	25.27a	74.82c	118.75bc	86.54cd
12	0.033a	0.028ab	0.022ab	0.847a	0.777a	0.158c	6.5a	7.38a	2.28b	25.62b	26.95b				11.67a			24.96a	81.28d	90.7ef	110.19ab
14	0.031b	0.028b	0.022bc	0.764b	0.806a	0.716a	6.49a	7.54a	5.46a	31.43a	30.65a	24.8							95.89c	100.7de	55.98e

	% Pop A			% Pop C			% Pop D			% Pop E			% Pop E1			% Pop E2			% Pop F		
CT (min)	W	V	GF	W	V	GF	W	V	GF	W	V	GF	W	V	GF	W	V	GF	W	V	GF
0	83.98a	85.48a	85.23a							89.96a	90.52a				30.47f			65b	10.04a	9.48a	4.53b
1	74.4b	83.14b	78.42b	20.28a	40.18a	18.52a	54.27a		12.82a	23.34e	57.35d				51.48a			16.02h	2.11ef	2.47c	1.16e
2	70.48c	72.51c	78.19b	18.69b	20.35b	14.76b	34.91b	58.47a	12.27a	44.44d	19.44f				45.55b			26.04g	1.95ef	1.74e	1.38cd
5	59.73d	64.47d	63.19c	10.37c	13.84c	9.57c	12.83c	44.88b	9.68b		39.06e		39.4a		38.88d	35.56d		39.94f	1.84ef	2.23cd	1.93cd
6	59.43d	56.69e	63.77c	10.32c	9.38d	7.68d	10.27de	12.33cd	9.27b				32.86b	38.24a	41.13c	44.85c	38.29d	40.96f	1.71f	1.77e	0.96e
8	53.54e	50.72f	57.34d	8.26e	8.48de	6.56e	9.14ef	9.76ef	7.96c				25.04d	32.26b	34.01e	54.16a	47.57c	50.2e	3.22c	1.92de	1.27e
9	47.95f	49.04f	54.01e	7.62f	7.77def	5.17f	9.26ef	8.69f	8.06c				33.25b	32.03b	31.64f	47.84b	49.74bc	53.97d	2.03ef	1.76e	1.16e
10	45.32g	41.81g	51.46f	7.06g	7.21ef	5.09fg	8.33f	8.84f	6.76d				26.43c	29.78c	26.42g	55.62a	52.06a	59.51c	2.56de	2.11cde	2.22c
12	41.38h	39.5h	46.68g	9.09d	7.68def	4.07h	11.87cd	13.39c	5.83e	74.49c	75.54c				17.77h			71.23a	4.54b	3.39b	1.1e
14	37.41i	34.98i	41.58h	7.38fg	6.38f	4.6g	10.25de	11.17de	9.3b	79.3b	78.72b	80.77							3.08cd	3.73b	5.33a

Table 3. ^{1}H populations relaxation time and relative abundances. Different superscript letters indicate significant differences ($p \leq 0.05$).

STD	PCA	PLS	W	PCA	PLS	V	PCA	PLS	GF	PCA	PLS
Macro	MC		Macro	MC		Macro	MC		Macro	MC	
Meso	DG		Meso	DG		Meso	DG		Meso	DG	
	E'	E'		E'			E'			E'	E'
	E''	E''		E''			E''			E''	E''
	Tanδ			Tanδ			Tanδ			Tanδ	
Molecular	FIDTA	FIDTA	Molecular	FIDTA	FIDTA	Molecular	FIDTA	FIDTA	Molecular	FIDTA	FIDTA
	%PopA			%PopA			%PopA			%PopA	
	T_2C			T_2C	T_2C		T_2C	T_2C		T_2C	
	T_2D			T_2D			T_2D			T_2D	
					%PopD			%PopD			
	T_2E			T_2E			T_2E			T_2E	
	%PopE			%PopE	%PopE		%PopE	%PopE		%popE	
	T_2E1			T_2E1	T_2E1		T_2E1	T_2E1		T_2E1	
	%PopE1			%PopE1	%PopE1		%PopE1	%PopE1		%PopE1	%PopE1
	T_2E2			T_2E2	T_2E2		T_2E2	T_2E2		T_2E2	
	%PopE2			%PopE2	%PopE2		%PopE2	%PopE2		%popE2	%popE2
	T_2F	T_2F					T_2F			T_2F	
							%PopF			%PopF	

Table 4. Discriminant features for PCA and PLS of cooking quality parameters of different formulations.

Figure 1.

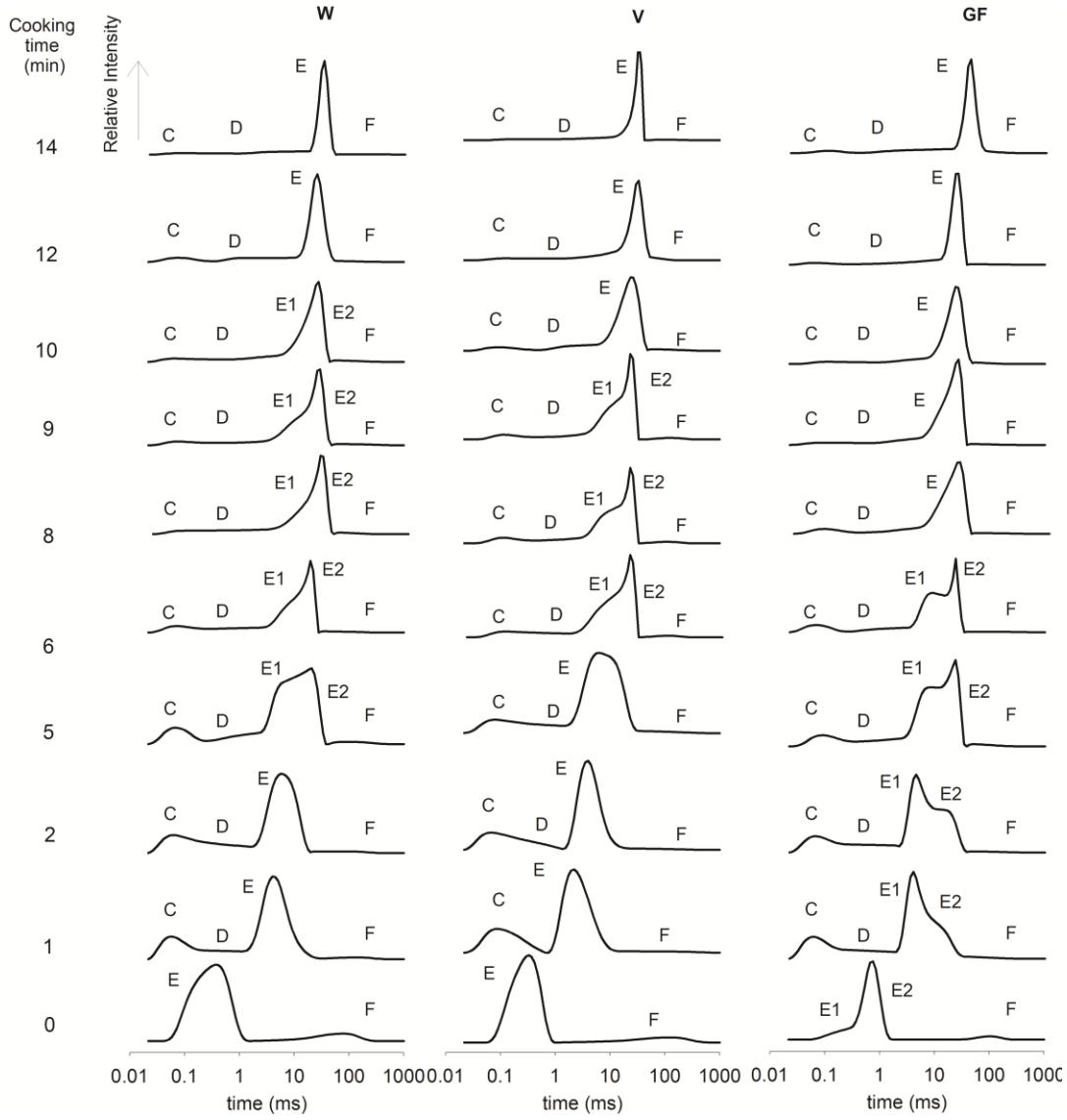
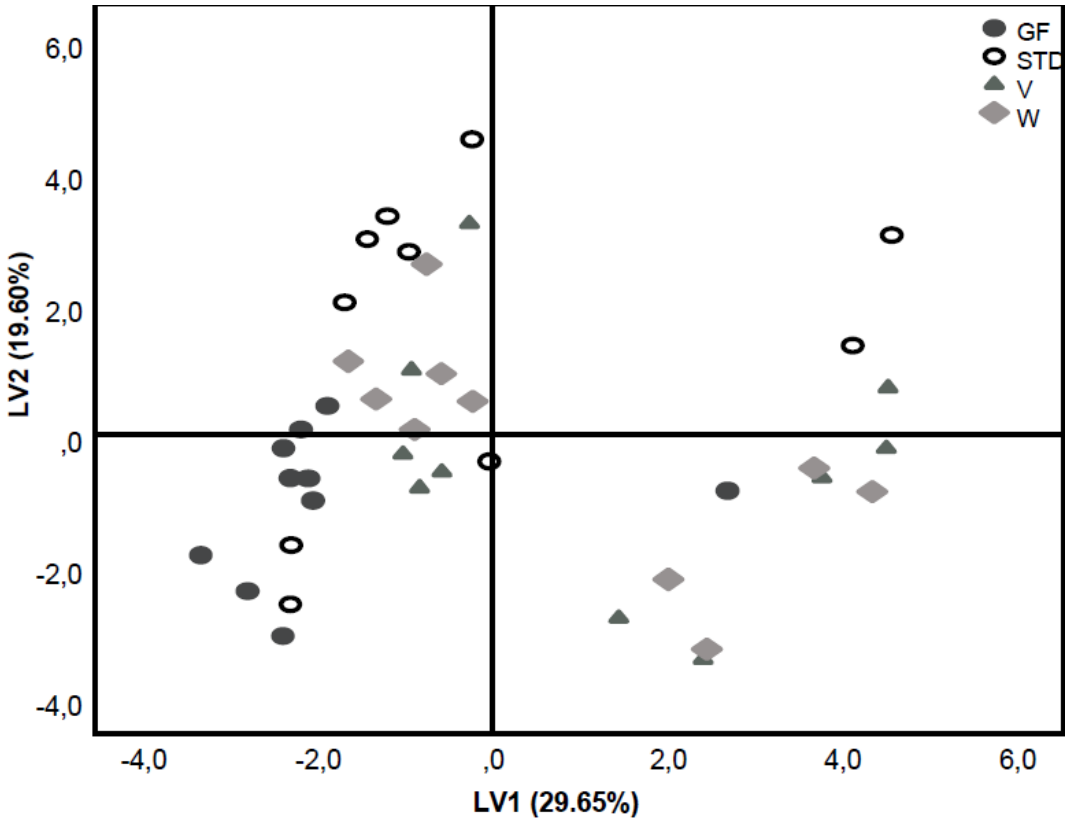

Cooking time (min)
Relative Intensity
W
V
GF
14
12
10
9
8
6
5
2
1
0
C
D
E
E1
E2
F
0.01
0.1
1
10
100
1000
time (ms)

Figure 2.



Legend to figures

Figure 1.

Representative ^{1}H T_2 quasi-continuous distributions over cooking of W, V and GF pasta during cooking.

Figure 2.

Partial Least Square for STD (○), W (♦), V (▲) and GF (●) pastas.

Section B: BREAD

Littardi et al., to be submitted

Two-dimensional NMR relaxometry: putting the puzzle together to decipher molecular dynamics in gluten-free bread during staling?

Abstract

Molecular dynamics in glutenfree (GF) bread staling are not yet well elucidated. To decipher this complex phenomenon, beside conventional methods, an innovative NMR application (two-dimensional 2D T_1-T_2_Corr) was applied toghether with one-dimensional ^{1}H NMR (Free Induction Decay, FID; proton T_1 relaxation time, $^1HT_1$; proton T_2 relaxation time, $^1HT_2$). Two GF bread formulations were throughly characterized. Conventional assessment showed the already known trend in the evolution of this parameters. As for molecular dynamics, 1D-NMR investigation in GF breads showed the presence of 4 and partially overlapped proton populations, while 2D-NMR provided better resolution of proton peaks. Moreover, it depicted much more complex interactions among bread components within the bread matrix. Even though 2D-NMR allowed a better understanding of different peaks belonging to different protons domains in STD bread, the high complexity of data in GF formulations still requires further studies for peak assignments and full understanding of the phenomenon.

Keywords: bread, gluten free, staling, 1D Nuclear Magnetic Resonance (NMR), 2D T_1-T_2 correlation NMR

Introduction

Due to the increasing prevalence of celiac disease (around 1–2 % of the population) together with consumers following gluten-free (GF) diet as a lifestyle, the market of GF products is witnessing a continious expansion. GF bread is known to have a high tendancy to shorten shelf life and a lower quality with respect to white wheat bread (Purhagen *et al.,* 2012; Ronda and Roos, 2011). Such a bread has also a more marked firmness increase during storage, resulting from the high amount of starch included in their formulations (Gallagher *et al.,* 2004; Osella *et al.,* 2005; Sciarini *et al.,* 2010; Ziobro *et al.,* 2013). Particularly, the relevant amylopectin retrogradation induces modification of structure and textural attributes of the aging product (Moore *et al.,* 2004; Cappa *et al.,* 2013; Capriles and Arêas, 2014; Demirkesen *et al.,* 2014). It is well known that gluten has a central role in the determination of structural bread quality, while this role in GF formulations is fulfill by the starch fractions (Veraverbeke *et al.,* 2002). Therefore, it is necessary to understand the staling phenomenon to find adequate solution to improve the quality of GF bread during storage.

During storage, molecular dynamics are mainly related to the high moisture content of GF breads with respect to regular bread due to the larger number of water molecules available for macroscopic migration as well as the absence of gluten (Gallagher *et al.,* 2004; Masure *et al.,* 2016). These conditions obviously favour all staling-related phenomena. Even though the molecular dynamics occurring in regular bread during storage has been extensively studied with the implementation of Time Domain ^{1}H Nuclear Magnetic Resonance, TD-NMR, (Wang *et al.,* 2004; Sereno *et al.,* 2007; Curti *et al.,* 2011; Bosmans *et al.,* 2013; Curti *et al.,* 2015; Curti *et al.,* 2017a;), they are not completely elucidated. In the case of GF, TD-NMR investigation is in its early stages, and only few works are available in this concern

(Purhagen *et al.,* 2012; Hager *et al.,* 2014; Carini *et al.,* 2017). Generally, they observed a complex relaxation behaviour, with multiple and partially overlapping proton populations (similar to regular bread), which leaves lot of room for speculation (Bosmans *et al.,* 2012; Bosmans *et al.,* 2013). Indeed, the complexity of GF bread formulations, where a variety of ingredient is used to mimic the gluten performance and create a structure resembling regular bread, makes the investigation of staling in GF bread a very challenging task (Moore *et al.,* 2004; Ahlborn *et al.,* 2005; Zannini *et al.,* 2012).

For this reason, a multi-analytical approach is indeed needed to understand bread staling as different physico-chemical phenomena occur at different structural levels (Gray and Bemiller, 2003). Innovative tools such as two-dimensional (2D) methods NMR (low and high resolution) have been applied to different food products (Van Duynhoven *et al.,* 2010; Kovrlija *et al.,* 2017) showing great ability to provide a better resolution of peaks similar to those of one-dimensional relaxation times (Hills *et al.,* 2004; Marigheto *et al.,* 2008; Furfaro *et al.,* 2009a, 2009b;). As well, low resolution 2D (T_1-T_2) NMR has been previously applied to cereal products (cake and corresponding model systems, Luyts *et al.,* 2013; dough and biscuits, Serial *et al.,* 2016; starch – water systems, Kovrlija & Rondeau-Mouro, 2017). In the case of bread, only recently Curti *et al.,* 2017b, investigated regular bread staling using 2D (T_1-T_2) NMR relaxometry indicating that such a tool enabled a better resolution of relaxation phenomena. This work aims, therefore, to apply conventional and innovative methods (i.e. 2D (T_1-T_2) NMR relaxometry) to enbable a deeper undresanding of molecular dynamics of GF bread staling, in comparison with a STD bread (Curti *et al.,* 2017b).

Material and methods

Bread formulation

Two GF breads were produced using two different formulations. For bread GF1, a commercial GF bread flours mix was purchased in a local supermarket and produced, following the producer instructions: commercial mix (52.9%), bottled water (42.3%), sunflower oil (2.1%), brewer's yeast (1.6%), salt (1.1%) Mix ingredients were the following: corn starch, rice flour, tapioca starch, sugar, salt, guard flour and E464, yeast extract.

Bread GF2 was produced using the following industrial recipe: bottled water (45.6%), corn starch (23.8%), potato flour (9.4%), Hi-Nucleus BRDM03 (8.1%), rice flour (6.7%), sunflower oil (2.4%), sugar (1.6%), brewer's yeast (1.0%), salt (1.0%), calcium propionate (0.5%). Water (Acqua Minerale San Benedetto S.p.a., Venezia, Italy), sunflower oil (Oleificio Zucchi, Cremona, Italy), sugar (Coprob S.C.A, Pavia, Italy), brewer's yeast (AB Mauri Italy S.p.a., Casteggio, PV, Italy) and salt (Italkali s.p.a., Palermo, Italy) were purchased at a local supermarket, while all other ingredients were supplied by Hi-Food (Parma, Italy). Hi-Nucleus BRDM03 was composed by: vegetable fibers, thickener: E 464, legumes flours, natural flavours, whole flaxseed meal, millet flour, buckwheat flour; processing aid: enzymatic preparations (transglutaminase).

Also, a conventional bread was taken in consideration as standard (STD) where its characterization was performed in previous work (Curti *et al.,* 2017b).

Bread production and storage

Two home bread-maker (Backmeister 68511, UNHOLD, Germany) were used to produced 4 bread loaves for each production employing a "personalized" program (kneading 25 min; proofing 42 min; baking 55 min) using water previously heated at about 35°C. Bread

loaves were cooled down for 2.5 h at room temperature and then analysed fresh (day 0) and after 1, 3, 5 and 7 days of storage (loaves placed in polyethylene bags sprinkled with ~4 ml of ethanol and stored at room temperature). At least two bread productions were carried out on different days for each storage time. STD bread loaves were produced using a ‘‘basic” program (preheating 17 min; first kneading 5 min; second kneading 13 min; first fermentation 45 min; smoothing 1 min; second fermentation 18 min; smoothing 1 min; third fermentation 45 min; baking 55 min) (Curti *et al.,* 2017b).

Conventional assessment

Bread composition

Bread composition was determined in both formulations by an external laboratory in terms of total starch content (using Megazyme Total Starch Assay Kit), proteins (AACC Official Method 46- 11), lipids (AOAC 935.38) and fibres (AOAC 985.29). Both GF formulations showed a total starch content ~74%, but GF1 had ~2.4 % proteins, ~2.5% lipids and ~4.4% fibres; while GF2 had ~ 1% proteins, 0.9% lipids and 2.9% fibers.
Also, STD bread composition was determined: 6.6% proteins, 1.5% lipids, 2.6% fibres.

Moisture content

Moisture content (MC) (%, g water / 100 g sample) of crumb (from loaf centre) was determined by weight loss by oven drying (NSV 9035, ISCO, Milan, Italy) at 105 °C to constant weight. At least triplicate samples of crumb were analysed for each bread loaf.

Texture

Bread crumb texture was measured using a TA.XTPlus Texture Analyzer (Stable Micro Systems, Goldalming, UK). At least 10 cubic portions (8 cm^3) of crumb were extracted from the central slices of each bread loaf and compressed with a TPA test (force = 0.05 N, 40% deformation, cylindrical probe - P/35 Dia Cylinder Aluminium) measuring hardness, cohesiveness and springiness.

Thermal analysis

A Q100 Differential Scanning Calorimeter (TA Instruments, New Castle, DE, USA) was used to measure thermal properties of breads. The instrument was calibrated with indium (melting point: 156.6 °C, melting enthalpy: 28.71 J/g) and mercury (melting point: -38.83°C, melting enthalpy: 11.44 J/g). About 4 g of crumb from loaf centre were compressed with a 2.5 kg weight to obtain a flat and compact specimen to maximize heat transfer during the experiment. Compressed bread crumb samples (5-10 mg) were taken and placed in hermetic stainless steel pans (Perkin Elmer, Waltham, MA, USA) and heated from -80°C to 130°C at 5°C / min. DSC data were analysed with a Universal Analysis Software (v. 3.9A, TA Instruments, New Castle, DE, USA) to measure the melting enthalpy of ice (from -20 to +15°C), to calculate the frozen water content, FW (Curti et al., 2014); while retrograded amylopectin, RA (50-80°C) was normalized on the basis of the starch content.

Molecular characterization

Nuclear Magnetic Resonance (^{1}H NMR)

^{1}H mobility was investigated with a low-resolution NMR spectrometer (20 MHz, the miniSpec, Bruker Biospin, Milano, Italy) operating at 25.0 ± 0.1°C. About 1.5 g of bread

crumb (taken from the bread loaf centre) were placed into a 10 mm NMR tube, compressed, sealed with Parafilm® (to prevent moisture loss during the NMR experiment) and analysed. For all experiments the recycle delay was 1 s (≥ 5 T_1), and 90° and 180° pulse lengths were adjusted for each sample.

1D Relaxometry. ^{1}H Free Induction Decay (FID) experiment was performed applying a single 90° pulse (scans: 32, dwell time: 7μs, acquisition window 0.5 ms). A two-component model (exponential and Gaussian; Le Grand *et al.,* 2007)

$$F\ (t)\colon y0 + A * exp(-t\ /\ T_A\ ) + B *\ exp[-(t\ /\ T_B\ )^2]$$

(where *y0* is the FID decay offset, *A* and *B* are the intensities of each relaxation component, T_A and T_B the apparent relaxation times) was used to fit the experimental curves (Sigmaplot, v10, Systat Software Inc. USA). ^{1}H longitudinal relaxation time (^{1}H T_1) curves were acquired with an Inversion Recovery pulse sequence, with 4 scans, a log-spaced inter pulse from 0.1 ms to 4000 ms and 20 data points.

^{1}H transverse relaxation time (^{1}H T_2) experiment was carried out with a CPMG pulse sequence, with an interpulse spacing of 0.04 ms, 32 scans and 4000 data points. Quasi-continuous distributions of relaxation times were obtained using the UPENWin software (Alma Mater Studiorum, Bologna, Italy), setting up default values for all UPEN parameters (with the exception of LoXtrap, that was set to 1 to avoid extrapolation of times shorter than the first experimental point). $^1HT_2$ curves were also fit with discrete exponential models (Sigmaplot, v.10, Systat Software Inc. USA) to obtain relaxation times and relative abundances of each proton population.

2D Relaxometry. T_1–T_2 data were acquired with a ^{1}H bi-dimensional T_1 – T_2 experiment (Bruker BioSpin GmbH, Rheinstetten; Germany) kindly provided by Bruker for tests

purposes. The pulse sequence consists of an Inversion – Recovery (180 degrees pulse – delay t_i – 90 degrees pulse) followed by a CPMG train (tau – 180 degrees pulse – tau), whose echoes intensities are recorded.

Raw data were elaborated with a beta-version bi-dimensional Inverse Laplace Transformation software (Bruker BioSpin GmbH, Rheinstetten; Germany) to obtain the 2D T_1–T_2 maps and the characteristics relaxation times and integrated proton population abundances.

The T_1–T_2 matrixes were acquired using the following settings: 150 T_1 points, increment factor of 1.08, from 0.1 ms to 10 s, and CPMG (tau = 0.0565, 4000 echoes). Raw data were elaborated with a beta-version bi-dimensional Inverse Laplace Transformation software (Bruker BioSpin GmbH, Rheinstetten; Germany) to obtain the 2D T_1–T_2 maps and the characteristics relaxation time and integrated population abundances.

Statistical analysis

A one-way-analysis of variance (ANOVA) followed by Duncan's test were carried out to verify significant differences in the evaluated parameters during storage at $p \leq 0.05$. Correlation coefficients (r) were computed using Pearson's coefficient ($p \leq 0.05$). All analyses were performed using SPSS software (version 25.0, SPSS Inc., Chicago, IL, USA).

Results and Discussion

Conventional properties

A conventional bread, whose characterization was reported in Curti *et al.,*2017b, was considered in the present work as a standard (STD).

Physico-chemical changes occurring in both GF1 and GF2 during storage are reported in Figure 1. Crumb moisture content (MC, Figure 1b) was 48.7% (GF1) and 51.3% (GF2) in fresh bread and it decreased to 46.8% (GF1) and 50.6% (GF2) over storage. Differently, crust MC (Figure 1a) significantly increased from ~17% to ~29% in GF1 and from ~20% to ~30% in GF2. Crust and crumb MC of GF fomulations were always significantly higher (Table 1) than STD (with GF2 higher then GF1), reflecting the high level of water in GF2 recipe (Carini et al., 2017). Crumb hardness (Figure 1c) significantly increased (Hager et al., 2014) from day 0 (0.8N) to day 7 (6.8N) in GF1 and from 2.6N and 6.1N in GF2. Moreover, at day 0 and 1 GF1 was comparable to STD and significantly lower than GF2, at day 3 and 5 GF2 was the harder, followed by GF1 and STD, while at day 7 GF1 and GF2 showed similar hardness and it was higher than STD. Springiness (Figure 1d) slightly decreased from ~1, at day 0, to ~0.9, at day 7 in both GF formulations with negligible differences between GF1, GF2 and STD, while cohesiveness (Figure 1d) decreased from 0.67 to 0.64 in GF1 and from ~0.73 to 0.56 in GF2.

Frozen water, FW (%) (Figure 1e) was found to be approximately constant in GF2 (oscillating in the range 49-56%) but showing to have comparable values to STD, while it significantly decreased in GF1 from 48% to 28%, showing a different trend depending on formulation (Hager et al., 2014). Amylopectin retrogradation (Figure 1f) was observed to increase during storage from 1.7, at day 0, to 5.7 (J/g of starch), at day 7, in GF1 and from 0.9 to 4.6 (J/g of starch) in GF2. GF2 was significantly always the more retrograded formulation over storage as compared to GF2 and STD. All the described phenomena (crumb dehydration, crumb firming, frozen water decrease and amylopectin retrogradation increase; Fadda *et al.,* 2014) were observed more pronounced in GF product.

Molecular properties.

1D Relaxometry. Representative Free Induction Decay curves of bread crumb during storage for both GF1 and GF2 are reported in Figure 2a. Protons observed with this experiment were usually attributed to the more rigid protons in the system, as previously reported (Curti *et al.,* 2011; Bosmans *et al.,* 2016). The slope of the first part of curves increased in all the formulations indicating an increase rigidity of the systems. The relaxation times of proton population A decreased from 0.030-0.035 to 0.02 ms (especially between days 5 and 7 of storage) with STD always the more mobile, GF2 the less mobile and GF1 with intermediate values. The relative abundances of this population (%popA) increased significantly in all the formulation with STD always the more abundant (from 56%, at day0, to 67% at day 7) and GF1 the less abundant (from 42% to 58%).

Representative ^{1}H T_1 distributions of GF1 and GF2 from day 0 to day 7 are shown at left in Figure 3. ^{1}H T_1 lineshape indicated the presence of one resolved proton population at day 0 relaxing at ~ 150-180 ms (data not shown). With increasing storage time, only minor changes were detected, with a slight shift of the distribution towards longer relaxation times. GF2 was always the more mobile in respect to GF1 and STD (Table 1).

^{1}H T_2 distributions of relaxation times indicated the presence of four proton populations, C, D, E and F (Figure 3, at right). Multiple ^{1}H T_2 populations were already reported in other studies where TD-NMR was applied to GF bread (Carini *et al.,* 2017; Purhagen *et al.,* 2012; Hager *et al.,* 2014). In these works each population was attributed to specific proton domains, and a similar assignment was applied in this study: population C was related to protons of amorphous starch and protein in little contact with water, population D to gluten and exchanging protons of confined water, starch, and protein, population E to mobile

exchanging protons of water, starch, and protein in the formed gel network, and population F to lipid protons.

In fresh and stored GF bread, population C and population D overlapped and were not completely resolved. PopC accounted for 12% and 9%, respectively in GF1 and GF2, of total protons at day 0 and decreased to 10% and 8% at day 7. This population showed a slight decrease of relaxation time T_2C, between ~0.5 ms and ~0.3 ms (in GF2) during storage, while it remained approximately constant (~0.4 ms) in GF1 (Figure 4). STD was always the less mobile but more represented (Table 1).

T_2D increased in both formulations in the range between ~3 and ~4 ms, it was the more mobile in GF samples as compared to STD, while its abundance showed variation in the range 10-15% also in STD (Figure 4 and Table 1).

The majority of detectable protons belonged to population E. The relaxation time decreased in GF1 from 15 ms to 13 ms, while in GF2 from 19 ms to 15 ms during storage while its abundance was ~72-77% (Figure 4). GF2 and GF1 were (in this order) always the more mobile and more represented, as compared to STD, over storage (Table 1).

The last and more mobile protons' population F (~4-5%, T_2F ~90-100 ms) (Figure 4) seemed to reflect the different lipids content of the samples, with GF2 the more represented, STD with intermediate values and GF1 the less represented (Table 1).

2D Relaxometry. In figure 5 are reported the bi dimensional T_1-T_2 maps for GF1 and GF2 bread formulations during storage. The implementation of the 2D NMR application allowed to increase the resolution of peaks. Indeed, we could resolve even 6 (in GF1) or 8 (in GF2) populations as compared to the 4, not completely resolved, with $^1HT_2$ and the single one obtained with $^1HT_1$. Moreover, the larger number of resolved peaks, as compared to a STD bread (Curti *et al.,* 2017b) could be due to the multitude of different ingredients used in GF

recipies. In confermation of this, the evolution of proton populations was more complex in GF formulations than in STD, with the appearance and disappearance of peaks during storage, being indicative of complex dynamics in the proton mobility and exchange.

Putting the pieces of the puzzle together

The bi-dimensional parameters taken in consideration for each resolved peak were: relaxation times T_1 and T_2 at the maximum inthensity and the relative area. In the optic to try to attribute each resolved population to a specific «class» of protons to better understand the dynamics at molecular level during gluten free bread staling, all the studied (macroscopic, mesoscopic and molecular) parameters were subjected to Pearson's correlation analysis.

Considering STD bread (that was prepared only with wheat flour, water, sugar yeast, sunflower oil and salt**)**, it showed 5 and well-defined 2D peaks (Curti *et al.,* 2017b) during storage. Among the obtained correlations between some 2D-NMR (2D-T_1C, 2D-T_2C, 2D-T_2B, 2D-%PopB and 2D-%PopD) and texture parameters ($r \geq \pm 0.898$, $p \leq 0.05$), 2D peak C could be tentatively attributed to amorphous domain, 2D peak B to gluten and 2D peak D to lipids. Also, evaluating correlations between 2D-T_2C with FW ($r = 0.888$; $p \leq 0.05$) or between 2D-T_1C, 2D-T_2C, 2D-%PopD and 2D-%PopE with RA ($r \geq 0.899$; $p \leq 0.05$) a similar peak attribution could be confirmed.

Correlations between 1D-NMR (FID TA and PopA) and texture indicators ($r \geq \pm 0.910$, $p \leq 0.05$) or RA ($r \geq \pm 0.918$, $p \leq 0.05$) were found in both GF1 and GF2 formulations, suggesting the hypothetical attribution of this proton population to the retrograded amylopectin. Also, intersting correlations between 1D-population E and texture parameters

(r = ± 0.956, $p \leq 0.05$) could indicate the attribution of this population to the amorphous domain. Even though it was observed a multitude of significant correlations in GF1 between 2D-NMR parameters and other indicators belonging to different levels of investigation, these correlations were not confirmed in GF2. For this reason, univocal assignments were not possible in the case of GF breads, in wich the complexity of formulation and the presence of different components hindered peak attribution. In the optic to better understand the molecular dynamics in GF bread it could be interesting to support this investigation with the study of more bread formulations and/or model systems.

Conclusions

Physico-chemical and molecular changes (both 1D and 2D) were taken in consideration to investigate staling in gluten free bread with different formulation (commercial and industrial) as compared to a STD bread.

Physicochemical properties showed the already known trend in the evolution of conventional parameters.

As for molecular dynamics, 1D-NMR investigation in GF breads showed the presence of 4 and partially overlapped proton populations in both formulations. On the contrary, 2D-NMR provided better resolution than 1D-NMR of the changes occurring in molecular mobility of different structural domains in the two formulations. Moreover, it depicted much more complex interactions among bread components within the bread matrix. Even though 2D-NMR allowed a better understand of different peaks belonging to different protons domains in STD bread, the complexity of the data still requires more studies for understanding molecular dynamics in GF formulations.

For this reason, it could be of great interest to consider a model system such as gluten, starch (or mix of them) and wheat flour at different level of water (cooked and uncooked) with 2D-NMR to gain more knowlodge on proton dynamics thereby to attribute univocally the resolved peaks.

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Table 1.

Days	Crust MC			Crumb MC			Hardness			Springiness		
	STD	GF1	GF2	STD	GF1	GF2	STD	GF1	GF2	STD	GF1	GF2
0	c/B	d/B	d/A	a/C	a/B	a/A	e/B	e/B	a/A	a/A	a/AB	a/B
1	b/B	c/AB	c/A	ab/C	ab/B	a/A	d/B	d/B	d/A	a/A	a/A	ab/B
3	a/C	c/B	b/A	b/C	a/B	b/A	c/C	c/B	c/A	a/A	a/A	ab/A
5	a/B	b/B	a/A	b/C	bc/B	ab/A	b/C	b/B	b/A	b/A	b/A	b/B
7	a/B	a/A	ab/A	c/C	c/B	b/A	a/B	a/A	a/A	b/A	b/A	ab/A
Days	Cohesiveness			Frozen water			Retrogr. Amylop.			^{1}H T_1		
	STD	GF1	GF2	STD	GF1	GF2	STD	GF1	GF2	STD	GF1	GF2
0	a/B	a/A	a/C	a/A	a/B	b/AB	-	d/A	c/B	a/C	c/B	c/A
1	b/B	b/A	b/C	a/A	a/B	a/A	c/B	cd/A	c/B	a/C	c/B	ab/A
3	c/A	c/A	c/B	a/AB	a/A	b/A	b/B	c/A	b/A	b/C	b/B	a/A
5	d/A	e/A	cd/A	a/A	a/B	a/A	a/A	b/A	ab/A	b/C	b/B	b/A
7	e/B	d/A	d/B	b/A	b/B	b/A	a/C	a/A	a/B	b/C	a/B	b/A
Days	^{1}H T_2C			^{1}H T_2D			^{1}H T_2E			^{1}H T_2F		
	STD	GF1	GF2	STD	GF1	GF2	STD	GF1	GF2	STD	GF1	GF2
0	b/C	a/B	a/A	b/C	c/B	c/A	b/C	a/B	a/A	a/B	ab/A	b/B
1	a/C	a/A	b/B	a/C	b/B	b/A	a/C	b/B	b/A	b/C	ab/A	ab/B
3	b/C	a/A	c/B	b/B	a/A	a/A	c/C	b/B	c/A	d/B	ab/A	a/A
5	b/C	a/A	d/B	b/B	a/A	ab/A	c/C	c/B	d/A	c/C	a/A	ab/B
7	b/C	a/A	d/B	ab/B	a/A	b/A	c/C	c/B	e/A	d/C	b/A	ab/B
Days	PopC			PopD			PopE			PopF		
	STD	GF1	GF2	STD	GF1	GF2	STD	GF1	GF2	STD	GF1	GF2
0	a/A	a/B	a/C	b/C	c/A	a/B	b/C	a/B	a/A	a/B	c/C	c/A
1	b/A	b/B	c/C	a/B	bc/A	a/B	a/C	b/B	b/A	c/B	c/C	a/A
3	c/A	c/B	c/C	b/B	a/A	a/B	c/C	b/B	c/A	b/B	b/C	b/A
5	c/A	c/B	b/C	b/C	a/A	a/B	c/C	c/B	d/A	d/B	b/C	b/A
7	c/A	c/B	bc/C	b/C	b/A	a/B	c/C	b/B	e/A	b/B	a/C	ab/A

Table 1. Different small or capital letters indicate significant differences ($p \leq 0.05$) between different days of storage or formulations, respectively. STD was taken from Curti *et al.,* 2017b.

Figure 1.

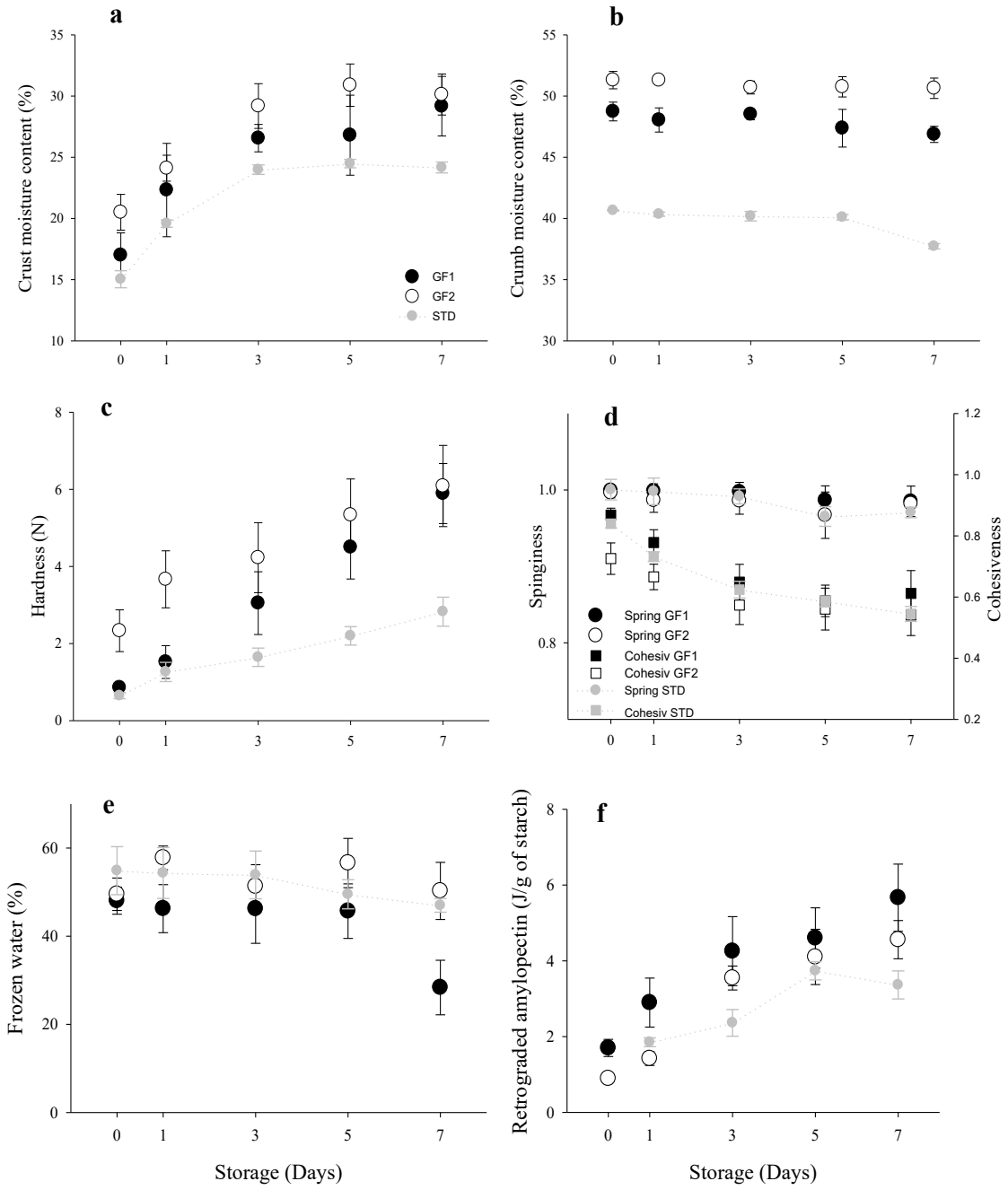

a
Crust moisture content (%)
GF1
GF2
STD
b
Crumb moisture content (%)
c
Hardness (N)
d
Spinginess
Cohesiveness
Spring GF1
Spring GF2
Cohesiv GF1
Cohesiv GF2
Spring STD
Cohesiv STD
e
Frozen water (%)
Storage (Days)
f
Retrograded amylopectin (J/g of starch)
Storage (Days)

Figure 2.

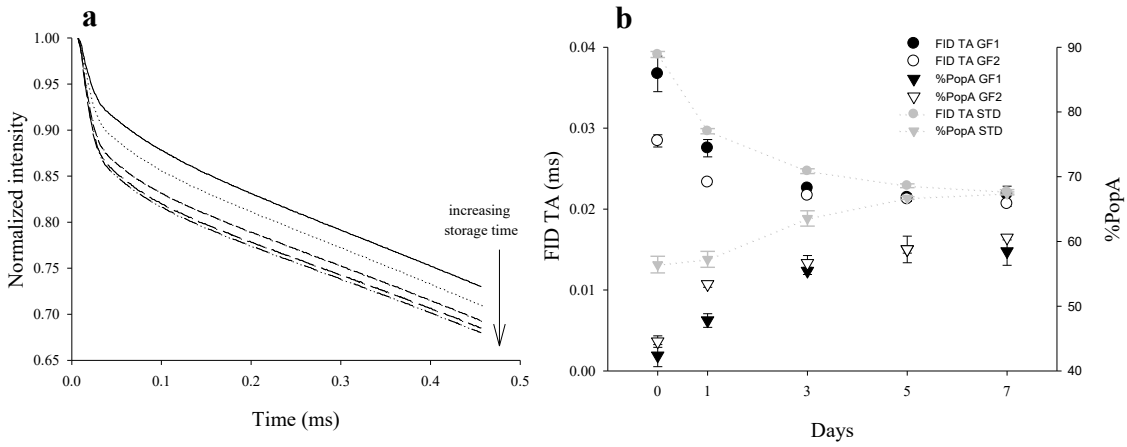

a
Normalized intensity
Time (ms)
increasing
storage time
b
FID TA (ms)
%PopA
Days
FID TA GF1
FID TA GF2
%PopA GF1
%PopA GF2
FID TA STD
%PopA STD

Figure 3.

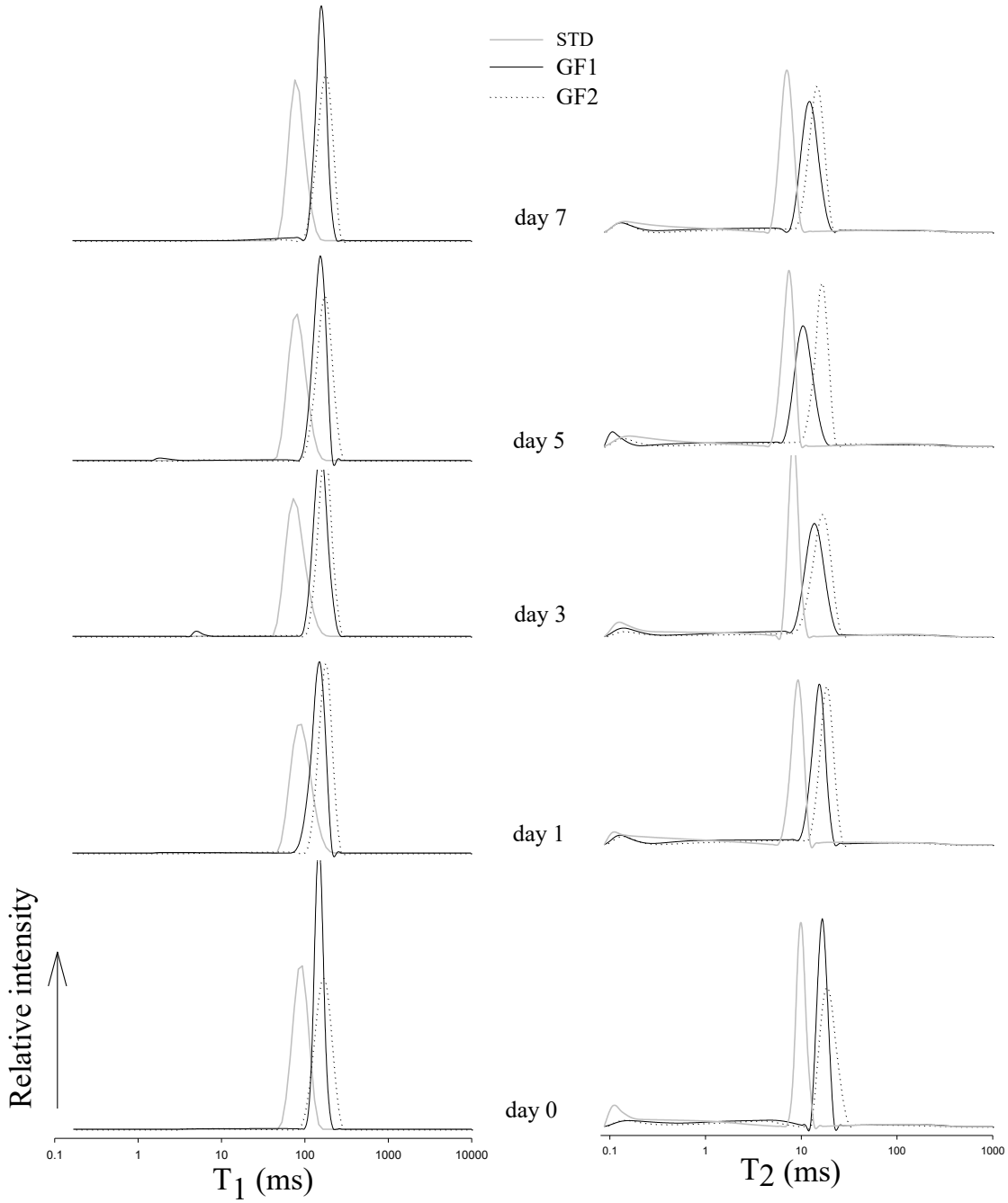
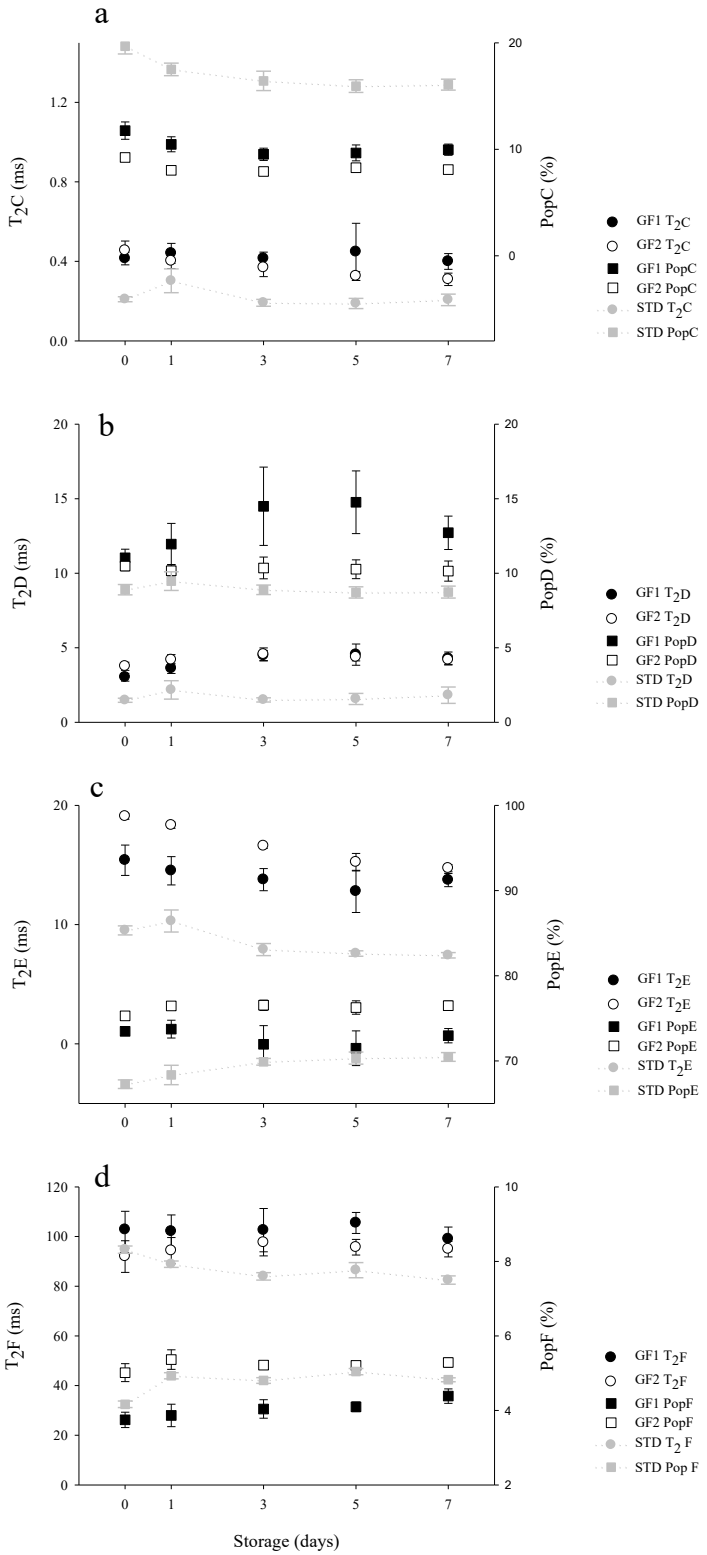

STD
GF1
GF2
day 7
day 5
day 3
day 1
day 0
Relative intensity
0.1
1
10
100
1000
10000
T1 (ms)
0.1
1
10
100
1000
T2 (ms)

Figure 4.


a
b
c
d
T2C (ms)
PopC (%)
T2D (ms)
PopD (%)
T2E (ms)
PopE (%)
T2F (ms)
PopF (%)
Storage (days)
GF1 T2C
GF2 T2C
GF1 PopC
GF2 PopC
STD T2C
STD PopC
GF1 T2D
GF2 T2D
GF1 PopD
GF2 PopD
STD T2D
STD PopD
GF1 T2E
GF2 T2E
GF1 PopE
GF2 PopE
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STD PopE
GF1 T2F
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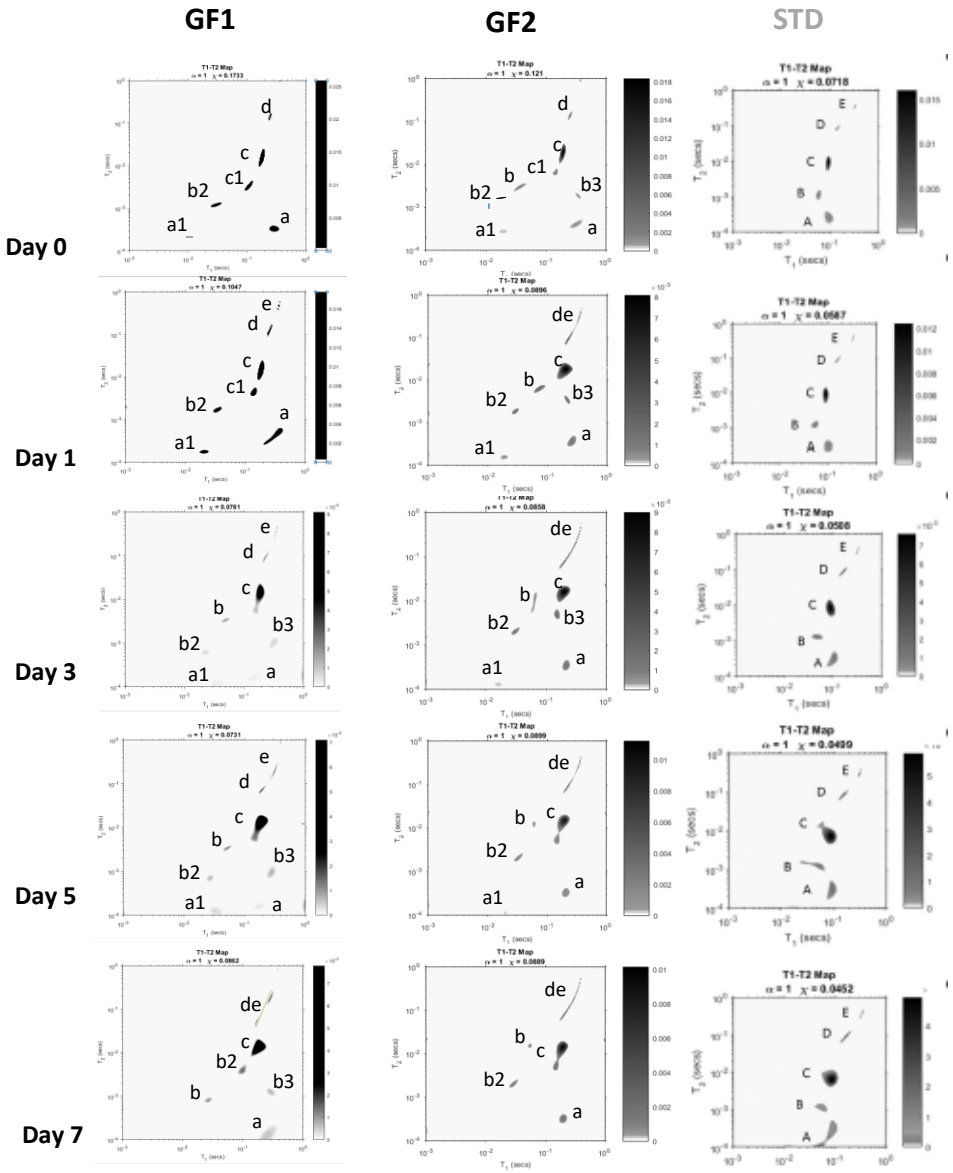
GF1 PopF
GF2 PopF
STD T2 F
STD Pop F

Figure 5.



Legends to Figures

Figure 1.

Crust (a) and crumb (b) moisture content hardness (c), springiness and cohesiveness (d), frozen water (e) and retrograded amylopectin (f) in breads GF1 (●), GF2 (○) and (●) STD (Curti et al., 2017b) during storage.

Figure 2.

Representative Free Induction Decay curves for all the formulations (a) with relaxation times of protons population A (FID TA, left axis) and relative abundances (%PopA, right axis)(b). Grey plots are referred to STD (Curti *et al.,* 2017b).

Figure 3.

^{1}H T_1 (left) and ^{1}H T_2 (right) distributions for breads GF1 (solid black line), GF2 (dotted black line) and STD (solid grey line; Curti et al., 2017b) during storage.

Figure 4.

Relaxation times (left axis) and relative abundances (right axis) of ^{1}H T_2 populations C (a), D (b), E (c) and F(d). Grey plots are referred to STD bread (Curti *et al.,* 2017b)

Figure 5.

Bi dimensional T_1-T_2_corr for GF1 (left) and GF2 (center) and STD (right; Curti *et al.,* 2017b) during storage.

Littardi et al., to be submitted

A multidimensional investigation: does β-amylase have an anti-staling effect on gluten-free bread?

Abstract

Preventing gluten-free bread staling with enzymes is proliferating practice in the food industry yet staling itself is not well deciphered from a multiscale approach. The focus of the present work was addressed to β-amylase (β-AMY) addition (0.5% and 1%) as a potential anti-staling agent with regard to a control [gluten-free industrial bread (0%)] during storage (from 0 to 7 days). In the specific, the macroscopic level was studied by means of textural properties, water content and water activity, the mesoscopic level by differential scanning calorimetry and dynamic mechanical analysis while the molecular level using low resolution time domain nuclear magnetic resonance. Based on multivariate statistics, storage time (ST) was the main factor influencing the macroscopic, mesoscopic and a part of molecular properties. Noteworthy, some molecular indicators (such as T_2C, T_2D, PopD, PopE, T_2F and PopF) were mainly influenced by the addition of β-AMY. The interaction (ST×β-AMY) also showed relevant effect on all studied parameters with the exception of springiness. Overall, considering each parameter separately, the enzyme addition did not show a significant anti-staling effect, yet some molecular features were the β-amylase seem to have a bland action.

Keywords: gluten-free, bread, 2D Nuclear Magnetic Resonance, Staling, Beta amylase

Introduction

One of the main concerns of the quality of gluten-free (GF) bread is its tendency to a rapid onset of staling due to the absence of gluten matrix and the large amount of gelatinized starch (Arendt & Dal Bello, 2011). To date, the food industry still calls to find solutions for preserving the quality of GF bread during storage. Subsequently, a multitude of solutions have been tested to prevent or limit the water migration and redistribution among component and the starch retrogradation in this bread. For instance, a variety of combination of GF cereals or pseudo-cereals flours and the addition of different proteins, hydrocolloids, as well as the sourdough fermentation have been largely used (Gallagher, 2009; Arendt & Dal Bello, 2011; Masure *et al.,* 2016).

Furthermore, enzymes deriving from raw materials or added in the formulation are intensively used as an anti-staling action in traditional white bread (Goesaert *et al.,* 2009; Bosmans *et al.,* 2013; Boukid *et al.,* 2018). Especially α-amylase showed great efficiency in the reduction of connections between starch crystallites (Hug-Iten *et al.,* 2003), lowering the tendency to form starch crystals. By definition, α-amylases are endo-amylases that random hydrolyse α-(1,4) glycosidic bonds of starch polymers, while β-amylases are eso-amylases acting at the non-reducing ends of starch chains, hydrolysing α-(1-4) glycosidic bonds, but not α-(1,6) stopping its activity at the branch point on amylopectin macromolecules (Goesart *et al.,* 2009). It was demonstrated that α-amylases' debranching action on amylose chains was found to be more pronounced than β-amylases for longer incubation time and for higher temperature (Bijttebier *et al.,* 2007). Therefore, α-amylases, having a drastic action on the starch chains, cannot be used in GF bread, where the gelatinized starch has an important structural role, as their action would result in crumb structural collapse. Indeed, β-amylase,

has a milder debranching action (Hug-Iten *et al.,* 2003), as it hydrolyzes the α-(1-4) glycosidic bonds with the release of maltose from the non-reducing end of starch chains, reducing the length of a part of the external chain. The presence of these small fragments hinders the reassociation of the long external chains (Wursch & Gumy, 1994). For this reason, it was hypothesized that β-amylases might provide a positive effect on GF bread staling while preserving structural integrity of the crumb. The aim of this work was, therefore, to explore, at different scale of investigation, the use of β-amylase as anti-staling agent in GF bread.

Material and methods

Ingredients and samples

The control sample (0%) was produced using the following recipe: bottled water (45.6%), corn starch (23.8%), potato flour (9.4%), Hi-Nucleus BRDM03 (8.1%), rice flour (6.7%), sunflower oil (2.4%), sugar (1.6%), brewer's yeast (1.0%), salt (1.0%), calcium propionate (0.5%). Water (Acqua Minerale San Benedetto S.p.a., Venezia, Italy), sunflower oil (Oleificio Zucchi, Cremona, Italy), sugar (Coprob S.C.A, Pavia, Italy), brewer's yeast (AB Mauri Italy S.p.a., Casteggio, PV, Italy) and salt (Italkali s.p.a., Palermo, Italy) were purchased at a local supermarket, while all other ingredients were supplied by Hi-Food (Parma, Italy). Hi-Nucleus BRDM03 was composed by: vegetable fibers, thickener: E 464, legumes flours, natural flavours, whole flaxseed meal, millet flour, buckwheat flour; processing aid: transglutaminase.

The 0% sample was compared to the same formulation with the addition of two level of a β-amylase (Hi-Softer 001). Hi-Softer 001 was a food enzyme preparation containing high

purity amylase of microbial origin with starch saccharifying activity ≈ 320 U/g. The carrier was maltodextrin from corn and tapioca. The suggested dosage of enzyme started from a minimum of 0.05% to a maximum of 2%, referred to the total dough (dry and liquid), depending on the product. Preliminary trials imparted that level of addition lower than 0.5% were not adequate to study the effect of enzyme addition, while at 2% it was observed the collapse of the structure. For these reasons, the selected levels of β-amylase addition were 0.5% and 1%.

Bread production and storage

Two home bread-maker (Backmeister 68511, UNHOLD, Germany) were used to produced 4 bread loaves employing a "personalized" program (kneading 25 min; proofing 42 min; baking 55 min) using water previously heated at about 35°C. After a 2.5 h cooling down at room temperature, fresh bread (day 0) was analysed, while loaves placed in polyethylene bags sprinkled with about 4 ml of ethanol and stored at room temperature were analysed during storage at 1, 3, 5, 7 days. At least two bread productions were carried out for each storage time.

Macroscopic properties

Moisture content

Moisture content (MC) (%, g water / 100 g sample) of crust and crumb (from loaf centre) was determined by weight loss by oven drying (NSV 9035, ISCO, Milan, Italy) at 105 °C to constant weight. At least triplicate samples of crumb were analysed for each bread loaf.

Water activity

Bread crust and crumb were broken into small pieces immediately before water activity measurement. Water activity was measured at 25°C with a Decagon Aqualab meter 127 TE8255 (Pullman, WA). Two samples both of crust and crumb (~3 g) were taken from two different bread loaves for a total of four measurements for each day of storage.

Texture

Bread crumb texture was measured using a TA.XTPlus Texture Analyzer (Stable Micro Systems, Goldalming, UK). At least six 8 cm^3 cubic portions of crumb, extracted from the central slices of each bread loaf, were compressed using a cylindrical probe (P/35 Dia Cylinder Aluminium) with a TPA test (force = 0.05 N, 40% deformation) measuring hardness, cohesiveness and springiness.

Mesoscopic properties

Thermal properties

The thermal properties of bread crumb were measured using a Q100 Differential Scanning Calorimeter (TA Instruments, New Castle, DE, USA) after calibration with indium (melting point: 156.6 °C, melting enthalpy: 28.71 J/g) and mercury (melting point: -38.83°C, melting enthalpy: 11.44 J/g). About 4 g of crumb from loaf centre were compressed with a 2.5 kg weight to obtain a flat and compact specimen with the purpose of maximize heat transfer during the experiment. 5-10 mg of the compressed crumb were placed in hermetic stainless steel pans (Perkin Elmer, Waltham, MA, USA) and heated from -80°C to 130°C at 5°C / min. DSC data were analysed with a Universal Analysis Software (v. 3.9A, TA Instruments, New Castle, DE, USA) to measure the melting enthalpy of ice (from -20 to +15°C) and retrograded amylopectin, (50-80°C). Frozen water content (FW, at the given experimental

conditions) was calculated, as previously described (Vodovotz *et al.,* 1996; Baik & Chinachoti, 2001; Curti *et al.,* 2014) using the following equation:

$$FW = Enthalpy\ Ice\ Fusion * \left(\frac{1}{Latent\ heat\ ice\ fusion}\right) * \left(\frac{1}{MC}\right) * 100$$

where FW is frozen water (%, g frozen water/ 100g water), enthalpy ice fusion (J/g product), latent heat of ice fusion is 334 J/g ice and MC is moisture content (%, g water/g product). Retrograded amylopectin was normalized on the basis of the starch content.

Viscoelastic properties

Dynamic mechanical properties [Storage modulus (E'), loss modulus (E'') and phase angle (tan δ, described as the ratio E''/E')] were measured using a dynamic mechanical analyser (DMA – Q800, TA Instruments, New Castle, DE, USA), by developing a tailored method for bread crumb. The central slice of the bread loaf (thickness ~ 1.2 cm) was compressed, after elimination of the crust, under 11 kg for 3.5 h in a sealed PE bag. A small cylinder of bread (~12 mm diameter and 3-8 mm thickness) was extracted from bread the compressed loaf and analysed in compression mode (15 mm diameter parallel plate compression clamp) with a frequency sweep test (1-40 Hz) at 25°C (force: 0.012 mN; preload: 0.015 mN; amplitude: 15 μm; soak time: 0 min). The amplitude of the frequency sweep test (15 μm) was determined with a preliminary strain sweep test, within the linear viscoelastic region (LVR). At least 12 analysis for each sample for each batch. E', E" and tanδ values were compared at a single frequency (25 Hz).

Molecular properties

^{1}H mobility was investigated with a low-resolution NMR spectrometer (20 MHz, the miniSpec, Bruker Biospin, Milano, Italy) operating at 25.0 ± 0.1°C. About 1.5 g of bread

crumb (from the bread loaf centre) were placed into a 10 mm NMR tube, compressed and sealed with Parafilm® (to prevent moisture evaporation during the experiment) and analysed (recycle delay, RD = 1s; optimization of 90° and 180° pulse lengths adjusted for each tube). Free Induction Decay (FID) experiment was carried out applying a single 90° pulse (dwell time of 7 μs, RD=1s, acquisition window= 0.5ms and data points=900). FIDs curves were fitted with two-exponential model (Sigmaplot, v10, Systat Software Inc. USA), according to the following equation, where *y0* is the FID decay offset, *A* and *B* are the intensities of each relaxation component, T_A and T_B the apparent relaxation times:

$$F\ (t): y0 + A * exp(-t\ /\ T_A\ ) + B *\ exp[-(t\ /\ T_B\ )^2]$$

(exponential and Gaussian; Le Grand *et al.,* 2007).

The Inversion Recovery pulse sequence was used to carry out ^{1}H longitudinal relaxation time experiment (^{1}H T_1) applying the following set up: 4 scans, log-spaced inter pulse in the range 0.1 - 4000 ms and 20 data points. ^{1}H transverse relaxation time (^{1}H T_2) experiment was carried out with a CPMG pulse sequence (interpulse spacing of 0.04 ms, 32 scans and 4000 data points). Curves were analysed as quasi-continuous distributions of relaxation times using the UPENWin software (Alma Mater Studiorum, Bologna, Italy). To avoid extrapolation of times shorter than the first experimental point, the UPEN parameter LoXtrap was set to 1, while other parameters were set to the default values. FID and $^1HT_2$ curves were also fit with discrete exponential models (Sigmaplot, v.10, Systat Software Inc. USA) to obtain relaxation times and relative abundances of each proton population.

Statistical analysis

Two-way ANOVA was performed taking in consideration the two fixed factors storage time and level of β-amylase addition (ST and β-AMY). The partition of total variance of sum

squares was computed to evaluate the contribution of single factors and their interaction in the variability of each parameter. Analysis of variance (one-way ANOVA) was conducted to evaluate the effect of storage on each parameter for each bread formulation at a significance level of $\alpha = 0.05$. Significant differences among the mean values were calculated using Duncan's test ($p \leq 0.05$). All experimental data were analysed using the SPSS version 25.0 (SPSS Inc., Chicago, IL, USA).

Results and discussion

Macroscopic Properties

In the optic of evaluate the effect of addition of β-amylase and storage time of bread on the studied quality parameters, 2 ways-ANOVA results (Table 1) revealed that the evolution of most of the macroscopic parameters (crust moisture content and water activity, hardness, springiness and cohesiveness) were mainly influenced by the storage time (ST) (SS% in the range 67-90), while crumb moisture content and water activity were influenced by ST×β-AMY (SS% 53 and 70, respectively) (Table 1).

Crust moisture content increased (from ~20% to ~30%), while crumb slightly decreased (from ~ 51% to ~ 50%) during storage (from 0 to 7 days) in all the formulations, due to the evaporation of water from crust and migration from crumb to crust (Baik et al., 2001). (Table S1)

Crust's water activity slightly increased (from ~ 0.85-0.87, at day 0, to ~ 0.93-0.94, at day 7), while crumb remained approximately constant (~0.96-0.97) in all the formulations during storage, due to water migration from crust to crumb (Baik & Chinachoti, 2000) (Table S1).

Hardness increased significantly in all the formulations during storage from ~2-3 N, in fresh samples, to ~6-8 N, at 7 days, in concordance with previous works (Curti *et al.,* 2016).

Springiness slightly decreased in 0% samples (from 0.99 to 0.98), while β-AMY supplemented showed a more considerable decrease (from 0.99 to 0.95). Likewise, cohesiveness underwent significant modifications in all the formulations showing a decrease (from 0.7-0.8 to 0.5-0.6). Overall, the macroscopic properties did not show any particular trend or difference following the enzyme addition (Table S1).

Mesoscopic properties

Concerning the mesoscopic parameters, the evaluation of SS% revealed that the effect of ST was the most relevant, where SS% ranged between 43 and 87% [e.g. retrograded amylopectin (SS%: 87), loss modulus (SS%: 43) and tanδ (SS%: 66)], while ST×β-AMY predominantly influenced frozen water and storage modulus (SS%: 62 and 60, respectively; Table 1).

Frozen water (FW) of the control sample (0%) showed some variations during storage, while that of β-AMY supplemented breads (0.5% and 1%) remained constant. Such a result might indicate that FW was not influenced by the presence of the enzyme. The retrograded amylopectin slightly decreased in 0%, 0.5% and 1% samples during storage, but no significant differences were found when compared to the control.

The evolution of viscoelastic properties showed the decrease of storage modulus, loss modulus and tanδ during storage. E' decreased from 0.032 to 0.019 MPa in 0% sample; from 0.018 to 0.013 and from 0.026 to 0.020 MPa, respectively in 0.5% and 1% samples. E'' decreased from 0.0057 to 0.003 MPa in 0%; from 0.0034 to 0.017 MPa and from 0.0051 to 0.034 MPa, in 0.5% and 1% samples, respectively. Tanδ decreased from ~0.2 to ~0.13-0.17 in all samples, during storage.

Molecular properties

Considering the NMR molecular indicators, even though the effect of ST remained the more relevant for FID TA, %PopA, T_1, %PopC and T_2E, with SS% between 82 and 98%, the β-AMY effect assumed importance in the determination of others molecular indicators such as T_2C, T_2D, %PopD, %PopE, T_2F and %PopF, with SS% between 37 and 90%. (Table 1)

Free induction decay (FID) curves, usually attributed to the most rigid protons in the system (Curti *et al.,* 2011; Bosmans *et al.,* 2016) showed an increase of the steepness of the first part of plot for increasing days of storage in both breads without and with enzyme addition, due to the increasing rigidity of protons belonging to this population. The relaxation time of the first and more rigid population A (FID TA) decreased during storage from ~0.03, at day 0, to ~0.02 ms, at 7 days; while its relative abundance increased from ~44% to ~60%. (Table 3)

^{1}H relaxation T_1, known to be little informative about the molecular dynamics of the staling phenomenon (Roudout *et al.,* 1999; Curti *et al.,* 2011; Curti *et al.,* 2017), was confirmed to present a single resolved proton population, that underwent small variations during storage and between different formulations, seesawing in the range of ~ 168-178 ms. (Table 3)

^{1}H relaxation T_2 showed a more complex evolution of proton population over storage, with the presence of 4 (C, D, E and F) not completely resolved peaks with different mobility (proton distributions not shown). These peaks were previously attributed to different domains of starch, proteins and fibre in little contact or not with water (Bosmans *et al.,* 2012; Hager *et al.,* 2014; Carini *et al.,* 2017).

Relaxation times and relative abundances of each ^{1}H population were reported in Table 3.

T_2C, usually attributed in gluten free bread to rigid and CH protons of starch and proteins in little contact with water, decreased of 0.14ms during storage (from 0.45 ms to 0.31 ms, in

0% sample); while in enzyme supplemented samples it decreased only of 0.1ms (in 0.5% sample) and 0.08ms (in 1% sample), indicating a less considerable stiffness of this class of proton during storage, with significant differences between the control and beta amylase supplemented ones. The effect of β-amylase, on the contrary, was not evident on the relative abundance of protons belonging to population C, decreasing significantly in all samples from ~9% to ~8% (Table 1). Relaxation time of population D, previously attributed to fibers – proteins exchanging protons of confined water, starch and proteins, increased during storage in both 0% bread (from ~3.8 to ~4.2ms) and more consistently in β-amylase bread (from ~4 to ~5-6ms), also indicating significant differences for increasing level of enzyme addition. Moreover, also the relative abundance of population D (PopD) showed the effectiveness of the enzyme: in 0% bread it remained constant during storage, while in 0.5% and 1% samples it increased significantly (from 10.6 to 12.4% in 0% sample; from 10 to 18% in 1% sample). T_2E, attributed to mobile exchanging protons of water and macromolecules in the formed gel network, decreased significantly during storage in all the formulations from ~19-20 ms to 15-17ms but its relative abundance (PopD) slightly increased in 0% bread while it slightly decreased (from ~75 to ~74) in 0.5% formulation and consistently decreased (from ~74 to ~68%) in 1% formulation. Relaxation time of the last and more mobile population (T_2F), attributed to lipid protons, remained approximately constant in 0%, varying between 92 and 98 ms during storage, while in 0.5% and 1% samples decreased from 91-92 to 87-88ms. The relative abundance of this population was ~5-6% in all the formulations over storage and showed the effectiveness of the addition of β-amylase, especially in 1% samples. The behaviour of relaxation time and relative abundance suggested that they have to be considered together and not singularly in the evaluation of bread staling phenomena. Indeed, in the case of proton population E although it decreased in relaxation time, indicating

stiffening of this class of protons, its relative abundance decreased too, indicating that the more rigid protons are less represented, contributing to have a less marked staling phenomenon.

Conclusions

The multiscale investigation of β-amylase enzyme as anti-staling agent in GF bread enabled to draw interesting conclusions. First, ST (and ST*β-AMY) was the controlling factor of the macroscopic properties (crust and crumb MC and a_w, texture parameters) and mesoscopic (FW, RA, E', E''and tanδ). At a molecular level, some parameters obtained using TD-NMR (T_2C, T_2D, PopD, PopE, T_2F and PopF) seemed to be influenced by the addition of β-amylase indicating the effectiveness of the enzyme in retarding the staling phenomenon. Such findings suggested that TD-NMR was more efficient in detecting of β-amylase potential as anti-staling agent in GF bread over traditional indicators. To confirm such these results, the use of a bi dimensional NMR application (2D T_1-T_2 correlation), together with the bread production with industrial process and the study for longer storage time, could be useful tools to understand and elucidate the use of β-amylase in retarding the staling phenomenon.

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Table 1.

		MACROSCOPIC							MESOSCOPIC					MOLECULAR									
		Crust MC	Crumb MC	Crust a_w	Crumb a_w	Hard.	Spring.	Cohesiv.	FW	RA	E'	E''	Tanδ	FID TA	%FID A	T_1	T_2C	%PopC	T_2D	%PopD	T_2E	%PopE	T_2F	%PopF
ST	SS%	90.73	39.09	76.78	20.74	82.98	67.44	88.23	17.01	87.03	29.94	42.71	65.97	97.53	95.94	73.13	40.00	86.27	40.69	28.42	81.92	14.41	6.20	3.78
	sig	***	***	***	ns	***	***	***	ns	***	***	***	***	***	***	***	***	***	***	***	***	***	ns	***
β-AMY	SS%	1.74	8.23	2.75	9.07	2.54	2.10	5.61	21.44	0.71	10.47	29.29	1.54	0.80	1.05	6.64	52.27	2.44	45.92	36.60	16.42	58.49	76.33	89.58
	sig	**	ns	*	ns	***	ns	***	ns	ns	***	***	ns	***	***	**	***	*	***	***	***	***	***	***
ST*β-AMY	SS%	7.53	52.68	20.47	70.19	14.47	30.46	6.16	61.55	12.26	59.59	28.00	32.49	1.67	3.01	20.23	7.73	11.29	13.39	34.98	1.66	27.10	17.47	6.64
	sig	***	***	***	*	***	ns	***	*	***	***	***	***	***	***	***	**	***	***	***	***	***	**	***

Table1. F significance level and sum square percent of the studied parameters and their interactions effects (***: $p \leq 0.001$; SS: sum of squares; ST: storage time; β-AMY: β-amylase addition)

Table 2.

Storage (days)	MC crust (%)			MC Crumb			Aw crust		
	0	0.5	1	0	0.5	1	0	0.5	1
0	20.5d/A	19.5c/A	20.4d/A	51.3a/A	50.8a/AB	50.4b/B	0.874b/A	0.853c/A	0.87d/A
1	24.1c/C	28.4b/A	26.8c/B	51.3a/A	51a/A	51.2a/A	0.863b/B	0.933b/A	0.914c/A
3	29.2b/B	33a/A	29.2b/B	50.7b/B	50.9a/B	51.6a/A	0.937a/AB	0.949a/A	0.926bc/B
5	30.9a/B	29.3b/A	32.1a/B	50.8ab/A	51a/A	49.7c/B	0.941a/AB	0.931b/B	0.947a/A
7	30.1ab/B	30b/A	31.7a/A	50.6b/A	50.7a/A	50.2bc/A	0.934a/A	0.941ab/A	0.938ab/A

Storage (days)	Aw crumb			Hardness (N)			Springiness		
	0	0.5	1	0	0.5	1	0	0.5	1
0	0.972a/A	0.972a/A	0.973a/A	2.33e/B	2.86c/A	2.07e/B	0.996a/AB	0.995a/B	1a/A
1	0.931b/A	0.972a/A	0.973a/A	3.66d/A	2.02d/B	3.86d/A	0.987ab/B	0.997ab/A	0.993ab/AB
3	0.974a/A	0.973a/AB	0.971a/B	4.23c/B	3.99b/B	5.74b/A	0.986ab/A	0.982bc/A	0.978bc/A
5	0.972a/A	0.969a/A	0.97a/A	5.33b/B	6.4a/A	4.79c/B	0.967b/B	0.97c/AB	0.986c/A
7	0.973a/A	0.97a/A	0.964b/B	6.09a/B	6.02a/B	7.83a/A	0.982ab/A	0.95d/A	0.96d/A

Storage (days)	Cohesiveness			Frozen water (%)			Retr. Amyl. (J/gr of starch)		
	0	0.5	1	0	0.5	1	0	0.5	1
0	0.725a/B	0.753a/AB	0.782a/A	49.5b/B	53.4a/B	61.1a/A	0.89c/A	0.77c/A	0.53b/A
1	0.666a/B	0.746b/A	0.687b/B	57.8a/A	57a/A	55.8a/A	1.42c/A	1.94c/A	1.43b/A
3	0.574b/A	0.617c/A	0.589c/A	51.3b/AB	49.4a/B	58.9a/B	3.55b/A	1.73c/B	3.81a/A
5	0.561c/AB	0.522d/B	0.597c/B	56.6a/A	55.9a/A	54a/A	4.1ab/A	4.23b/A	3.1a/A
7	0.541c/B	0.544d/B	0.612c/A	50.3b/A	55.8a/A	53.7a/A	4.56a/B	6.01a/A	4.25a/B

Storage (days)	Storage modulus (MPa)			Loss modulus (MPa)			Tanδ		
	0	0.5	1	0	0.5	1	0	0.5	1
0	0.032a/A	0.018b/B	0.026a/A	0.0057a/A	0.0034a/B	0.0051a/A	0.2a/A	0.19a/A	0.2a/A
1	0.025b/B	0.206a/A	0.009c/C	0.0047b/A	0.0026b/B	0.0016c/C	0.2a/B	0.21a/A	0.17b/C
3	0.018c/A	0.014b/B	0.019b/A	0.0029c/A	0.0029ab/A	0.0033b/A	0.15b/C	0.2a/A	0.17b/B
5	0.02c/A	0.012b/B	0.02b/A	0.0034c/A	0.0018c/B	0.0034b/A	0.17b/A	0.15b/A	0.17b/A
7	0.019c/A	0.013b/B		0.003c/B	0.0017c/A		0.16b/A	0.13c/B	

Table 2. Macroscopic and mesoscopic parameters. Different small letters indicate significant differences during storage ($p \leq 0.05$) capital letters indicate significant differences among formulations ($p \leq 0.05$).

Table 3.

Storage (days)	FID TA (ms)			PopA (%)			T_1 (ms)		
	0	0.5	1	0	0.5	1	0	0.5	1
0	0.028a/B	0.029a/A	0.03a/A	44.5e/A	44.4d/A	44.3d/A	168.8c/A	168.9c/A	169.5c/A
1	0.023b/C	0.025b/A	0.024b/B	53.4d/A	49.2c/C	50.8c/B	177.1ab/A	173.1b/B	172.7b/B
3	0.022c/B	0.022c/A	0.022c/B	56.6c/A	54.8b/B	56.6b/A	177.9a/A	178.2a/A	177.7a/A
5	0.021d/B	0.021d/C	0.022c/A	58.8b/B	60.4a/A	56.3b/C	175.2b/A	176.6ab/A	176.1a/A
7	0.021e/B	0.021d/B	0.021d/A	60.6a/A	60.3a/B	59.5a/B	175.6b/A	176ab/A	168.5c/B

Storage (days)	T_2C (ms)			T_2D (ms)			T_2E (ms)		
	0	0.5	1	0	0.5	1	0	0.5	1
0	0.45a/B	0.5a/A	0.52a/A	3.75c/B	4.17b/A	4.14c/A	19.1a/B	20.3a/A	20.5a/A
1	0.4b/B	0.45b/A	0.48ab/A	4.18b/B	4.11b/B	4.84b/A	18.3b/B	19.2b/A	19.4b/A
3	0.37c/B	0.44b/A	0.48ab/A	4.55a/C	5.03a/B	5.88a/A	16.6c/C	17.7c/B	18.3c/A
5	0.33d/B	0.35c/B	0.47ab/A	4.37ab/C	4.97a/B	5.83a/A	15.2d/B	16.3d/A	16.8d/A
7	0.31d/B	0.4bc/A	0.44b/A	4.18b/C	5.38a/B	6.11a/A	14.7e/C	16.8d/A	16e/B

Storage (days)	T_2F (ms)			PopC (%)			PopD (%)		
	0	0.5	1	0	0.5	1	0	0.5	1
0	92b/A	91.8a/A	90.8a/A	9.23a/A	9.29a/A	9.61a/A	10.48a/A	10.62bc/A	9.91d/B
1	94.3ab/A	84.8b/B	84.9b/B	8.02c/B	8.77b/A	8.54b/A	10.16a/B	10.27c/B	10.83d/A
3	97.6a/A	88ab/B	85.9b/B	7.91c/A	8.03c/A	8.09c/A	10.35a/C	11.58ab/B	12.82c/A
5	95.7ab/A	87.5ab/B	86.4b/B	8.27b/A	8.19c/B	7.97c/B	10.27a/C	11.48ab/B	15.35b/A
7	94.9ab/A	86.4b/B	87.6ab/B	8.09bc/A	7.75c/B	8.27bc/A	10.14a/C	12.37a/B	17.67a/A

Storage (days)	PopE (%)			PopF (%)		
	0	0.5	1	0	0.5	1
0	75.28b/A	74.59ab/B	74.44a/B	5.01c/C	5.51c/B	6.04bc/A
1	76.45a/A	75.19a/B	74.51a/C	5.37a/C	5.77ab/B	6.12ab/A
3	76.53a/A	74.59ab/B	73.2b/C	5.22b/B	5.8ab/A	5.89b/A
5	76.26a/A	74.72ab/B	70.4c/C	5.21b/C	5.61bc/B	6.28a/A
7	76.49a/A	74.04b/B	68.18d/C	5.29ab/B	5.85a/A	5.88c/A

Table 3. Relaxation times and relative abundances of proton populations. Different small letters indicate significant differences during storage ($p \leq 0.05$) capital letters indicate significant differences among formulations ($p \leq 0.05$).

Overall conclusions

A multiscale approach was used to describe the pasta cooking process and gluten-free bread staling in terms of macroscopic, mesoscopic and molecular properties.

For the first part, different commercial dry pasta and gluten free bread type/formulations were taken in consideration and compared with standard products. The use of different methodology of investigation (traditional or innovative), together with multivariate statistics and/or discriminant analysis allowed to identify different stages during the pasta cooking. Moreover, it was possible to clearly differentiate gluten free pasta from gluten containing types. Even though all the studied parameters were able to contribute to describe in some way the cooking process, molecular mobility indicators and viscoelastic properties were the most descriptive parameters, making TD-NMR and DMA valid techniques to characterize pasta.

For the second part, the same approach was adopted to understand physico-chemical and molecular changes during GF-bread. Results revealed that molecular indicators, 1D and 2D NMR, were the most effective in describing the differences in the staling process in different formulations. In the specific, 2D-NMR provided better resolution than 1D-NMR of the changes occurring in molecular mobility of different structural domains in the different formulations. Furthermore, the investigation of the potential effect of β-amylase as anti-staling agent was conducted using the multiscale approach. The results showed that exclusively the molecular level seemed to be influenced by the addition of the enzyme indicating the effectiveness of the enzyme in retarding the staling phenomenon.

Overall, these findings suggested that TD-NMR (both 1D and especially 2D) were more efficient in detecting and deciphering a part of the mystery of molecular dynamics in products with different formulations.

For future perspective, going back to the basis for mapping 2D NMR though considering a model system can be of great importance. Further in-depth analysis are ongoing, where gluten, starch (or mix of them) and wheat flour at different level of water (cooked and uncooked) were investigated using 2D-NMR to gain more knowledge on proton dynamics thereby to attribute univocally the resolved peaks.