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# THE STATE OF WATER IN BREAD:

# EFFECT OF PROCESSING, FORMULATION AND STORAGE

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# TO MY FRIENDS

"...I WAS JUST GUESSING AT NUMBERS AND FIGURES...
...QUESTIONS OF SCIENCE, SCIENCE AND PROGRESS..."

# SUMMARY

Bread staling is defined as the "decreased consumer acceptance caused by changes in crumb other than those resulting from the action of spoilage organisms". Although bread staling has been studied for more than a century, this phenomenon is not completely understood. Bread staling is a complex phenomenon that involves many events occurring at macroscopic, macromolecular and molecular levels, including amylopectin retrogradation, water redistribution, and loss of plasticity of the gluten network.

A multi-dimensional and multi-analytical approach was applied in this work in an attempt to thoroughly characterize bread staling and to investigate the effect of processing and formulation on bread properties and stability. In particular, since water is recognised to play a very important role in the changes occurring in bread during storage, this study focused on the role of water status and water dynamics on bread staling.

The results indicated that processing conditions and formulation affected not only macroscopic, macromolecular and molecular properties of bread but also its stability. The water status and the water-solid interactions resulted to be a key factor in bread stability. A better understanding of bread staling phenomenon, in relation to the macroscopic and macromolecular properties, was achieved by means of $^{1}H$ NMR (molecular mobility) that provided useful information on molecular changes occurring in bread during storage. $^{1}H$ $T_1$ molecular mobility was also successfully studied with $^{1}H$ NMR Fast Field Cycling, that highlighted changes in $T_1$ relaxation processes that were never reported in bread staling studies.

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# Introduction

## 1- Bread staling

Staling is "a term which indicates decreasing consumer acceptance of bakery products caused by changes in crumb other than those resulting from the action of spoilage organisms" (Bechtel, Meisner and Bradley, 1953).

Although it has been studied for more than a century, the forming structures and interactions involved in bread staling are not yet completely understood. Fresh bread can be defined as an unstable, elastic, solid foam, the solid part of which contains a continuous phase and a discontinuous phase: the continuous phase is an elastic network of cross-linked gluten molecules and leached starch (primarily amylose) polymers both uncomplexed and complexed with polar lipid molecules and the discontinuous phase is made by entrapped, gelatinized, swollen, deformed starch granules (Gray and Bemiller, 2003).

The starch-phase is one of the factors involved in bread staling. It is well known that amylose and amylopectin undergo recrystallization. In particular, amylose recrystallitazion, due to its linear structure, begins just after the cooking process as bread is cooled to room temperature (Hoseney and Seib, 1978). Gelatinized amylopectin requires longer times to reorganize into crystalline structures. Some studies reported that the degree of recrystallization is strictly related to crumb hardening (Champenois, Della Valle, Plancho, Buléon and Colonna, 1995), although others found no correlation between an high degree of recrystallized amylopectin with the observation of an harder crumb (Hallberg and Chinachoti, 2002).

Water has been recognized to play an important role in bread staling as it is involved at macroscopic, macromolecular and molecular levels. At a macroscopic level, a migration of water takes place, due to the presence of a moisture gradient between the crumb and the crust, contributing to the hardening of the crumb. Also the molecular redistribution of water molecular must be taken in consideration, since many studies reported that the moisture transfer of water molecules from gluten to starch might be involved in the staling process: it has been hypothesized that the recrystallization of amylopectin may involve the incorporation of water molecules in the crystalline structures (Imberty and Perez, 1988). Water molecules may have been released from the gluten network, that, therefore, may have lost its original plasticization to some extent. Other studies reported that some gluten-

starch (Martin, Zeleznak and Hoseney, 1991; Martin and Hoseney, 1991) or starch-starch interactions may take place (Every, Gerrard, Gilpin, Ross and Newberry, 1998).

A scientific approach aiming to the understanding of staling phenomena should, therefore, attempt to relate the microstructure to physical and sensorial properties, but it is really hard to find scientific literature that clearly demonstrates the relation between microstructure and macroscopic properties of bread. It is obvious that no instrumental analytical method will completely measure or describe the degree of staling as perceived by the consumer (Sidhu, Al-Saqer and Al-Zenki, 1996). Texture and rheological techniques are basic and provide an objective evaluation of staling since the decrease in the acceptance of bread can be easily evaluated by measuring crumb firmness (hardness) (Willhoft, 1973), the most used and easily measurable parameter to describe bread staling. Other techniques allow to characterize bread properties at "smaller" scales in an attempt to relate the macroscopic properties with microstructure (Schiraldi and Fessas, 2001). Thermal analysis techniques such as Differential scanning calorimetry (DSC), thermogravimetry (TGA) and dynamical mechanical analyis analysis (DMA) along with X-Ray diffraction can provide basic information at a macromolecular level on water status, starch retrogradation and macromolecular mobility (Karim, Norziah and Seow, 2000). Microscopic techniques can be used to monitor crumb structural changes during storage. Vodovotz and Chinachoti (1998) used Confocal Laser Scanning Microscopy (CLSM), that allows to obtain 3D images and provide information on the crumb structure, and reported that no differences were observed between images of fresh and stored bread, indicating that the most important changes during storage occur at a molecular level.

In recent years the study of bread staling has been focusing on the changes occurring at molecular basis using, mainly, NMR techniques. These techniques, including solid-state proton NMR, deuterium NMR, $^{13}C$ NMR with cross polarization and magic-angle spinning (CP MAS), and pulsed NMR have been widely used to examine changes in molecular mobility during staling.

## 2- Water and food stability

It is well known that water has a very important role in defying microbial, enzymatic and chemical stability of food systems but it also affects texture, physical state and acceptability of food materials (e.g. it can act as a plasticizer). Most of processing operations are influenced by water compartmentalization and microscopic redistribution, which, in turn,

affects macroscopic properties and food functionality (Vittadini and Vodovotz, 2007). Water has also a critical role in the definition of all levels of food structure: at the molecular level it can interact with other molecules (through hydrogen bonds, hydrophobic interactions, …) and affects their structure, mobility, plasticity and functionality. At an ultrastructural level water can modulate the association/breakdown of macromolecules as well as the formation of natural assemblies. At a microstructural level, where colloidal phenomena predominate, the role of water is critical in the formation of droplets (e.g. emulsion), crystals (e.g. ice formation), air cells (e.g. foams), etc. Finally, all these structural interactions manifest themselves at a macrostructural level (Vittadini and Vodovotz, 2007).

Water behaviour and its motions in a food product can be driven by different "forces", such as moisture and water vapour pressure gradients and chemical potential differences. The migration and diffusion of water can strongly affect food stability and have many implications that are manifested at different time-scale ranges and interest most of the food instability phenomena including, for example, gel syneresis, ice crystals formation, microbial growth, browning reactions, oxidation of lipids, enzymes stability, gelatinization of starch, texture of dry and intermediate moisture foods.

Water in food is present not only in different physical states (e.g. liquid or solid) but also in a number of different molecular environments making the study of its state and distribution really difficult. Some analytical techniques are commonly used to get some basic information about water in food, such as moisture content determination and water activity measurement that measure averaged and long range water properties. Water activity has been widely used as an indicator for food stability but its use has been challenged (Franks, 1991). Glass transition concept and state diagrams of food matrices were then introduced (Roos, Karel and Kokini, 1996; Roos and Karel, 1991a, Roos and Karel, 1991b) basically highlighting the relation existing between the stability of a food matrix and its glass transition: a food product could be considered stable at the glassy state, since below the glass transition temperature molecules involved in deteriorative reactions are believed to be, essentially, immobilized. It has been, although, pointed out that the glass transition temperature approach in complex matrices (such as food materials) measures the system's "long term mobility" (e.g. of the macromolecules) while "shorter range – faster" motions (e.g. of water) are still taking place in the glassy state (Li, Dickinson and Chinachoti, 1998; Vittadini and Chinachoti, 2003). Hence further investigations on molecular dynamics and mobility beyond the "macroscopic" measurements of glass transition is needed. The

concept of molecular mobility and food stability has, therefore, been proposed as a better indicator/predictor for food stability (Slade and Levine, 1991; Roos et al., 1996).

Water mobility of a system can be analyzed at different space-time levels with different analytical techniques. Differential Scanning Calorimetry (DSC) allows to determine the ability of water to freeze and, therefore, it can provide an insight about the degree of interaction of water within the food matrix. NMR relaxometry can be used to investigate the mobility in a very short time/space and, therefore, provides information about water properties at molecular level. Water molecular mobility can be analyzed by $^1$H NMR spectroscopy, since proton nuclei is the most abundant NMR detectable species and its signal acquisition is relatively easy. Although $^1$H NMR spectroscopy is not a specific probe for water (Halle and Wennerstroem, 1981; Schmidt and Lai, 1991; Colquhoun and Goodfellow, 1994; Ruan and Chen, 2001), the mobility of a food components is strongly affected by water (that acts as polymers plasticizer, solvent for solutes,…) and the observed $^1$H NMR signal encompasses also the contribution of the other species closely interacting with water and the molecular dynamics existing among the different domains characterized by their own mobility. $^1$H NMR mobility is a very useful tool to better comprehend and interpret the results and the information obtained with the other water descriptors.

Multiple $^1$H NMR experiments can be used to investigate $^1$H mobility. Of particular interest are the determination of the Free Induction Decay (FID), longitudinal ($^1$H $T_1$) and transverse ($^1$H $T_2$) relaxation times and $^1$H self diffusion coefficient, that provide information on rotational and translational proton (water) motions. More complex $^1$H NMR techniques (two-dimensional $T_1$-$T_2$ correlation relaxometry) have been discussed (Hills, Benamira, Marigheto and Wright, 2004) and applied to complex food systems.

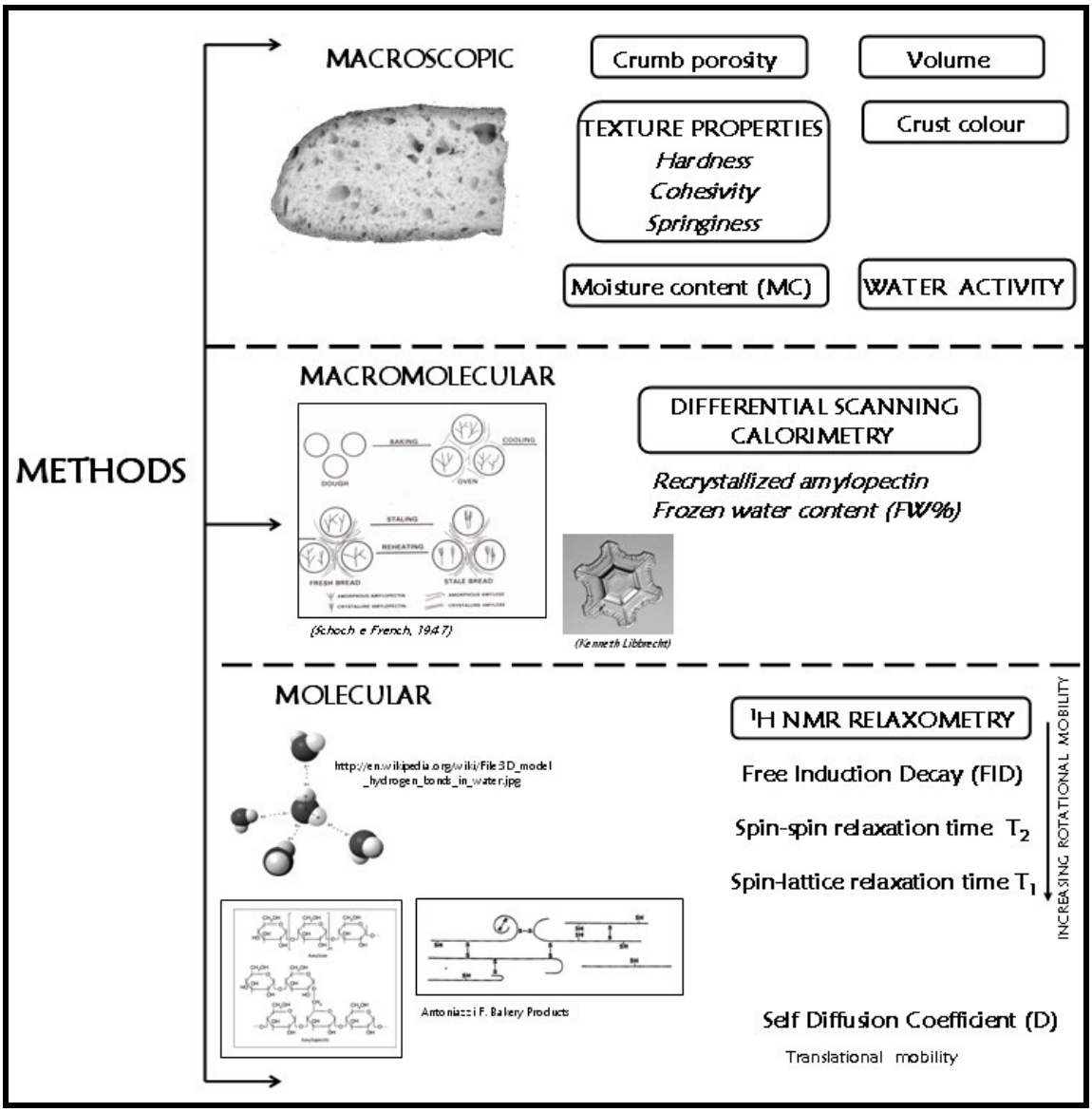
# Objective

The objective of this work was to characterize bread staling at multiple time-space scales, focusing, in particular, on the role of molecular mobility and water dynamics in this phenomenon. The effect of processing (e.g. mixing) and formulation (e.g. bran addition) on physico-chemical properties and stability of bread have also been evaluated.

# Methods of analysis

A multi-analytical approach was used in an attempt to relate macroscopic changes to molecular mobility. Particular attention has been given to the description of the water status with different parameters such as water activity, moisture content, "frozen water" content and $^1$H NMR mobility for a better understanding of properties and storage stability of bread in relation to formulation and processing.

The figure below shows a diagram and the description of the methods used for bread characterization throughout this thesis. Any modification to experimental settings will be reported in each section.



# 1. Macroscopic properties

## 1.1. Volume

Volume of bread loaves was measured following the American Association Cereal Chemistry 10-05 method (Guidelines for Measurement of Volume by Rapeseed Displacement). Two volume measurements were carried out for each sample.

## 1.2 Crumb Porosity

The crumb grain of the loaves was assessed using a digital image analysis system. Images of the three central slices (20mm thickness) from each loaf were captured with a flatbed scanner (Model Scanjet 8200, HP, Cupertino, USA), with a resolution of 600 dots per inch (dpi) and converted from true colour to 8 level grey scale. The images were processed using an Image-Pro Plus 4.5 (Media Cybernetics Inc., USA) software. Crumb grain was characterized by enumerating the pores present in five preselected dimensional classes based on their area.

## 1.3 Crust colour

Colour determination was carried out on the crust using a Minolta Colourimeter (CM 2600d, Minolta Co., Osaka, Japan) equipped with a standard illuminant D65. L* (lightness), a* (redness) and b* (yellowness) were quantified on each sample using a 10° position of the standard observer. ΔE was calculated [$= \sqrt{(L-L_0)^2+(a-a_0)^2+(b-b_0)^2}$] and STD values of L* a* b* were taken as the reference .

## 1.4 Texture properties

Bread crumb hardness, springiness and cohesivity were measured using with a TA.TX2 Texture Analyzer (Stable Micro Systems, Goldalming – UK). Cubic portions (2 x 2 x 2 cm3) of crumb were extracted from the central slices of the bread loaf and compressed (force = 0.1 N) to 40% deformation using a cylindrical probe (P/35 Dia Cylinder Aluminium). Hardness (height of the first compression peak), cohesiveness (ratio area second/first compression peaks) and springiness (ratio lenght second/first compression peaks) were determined. At least six replicates were run for each sample.

# 2. Macromolecular properties

## 1.2.1 Thermal analysis

Thermal properties were measured using a differential scanning calorimeter (DSC Q100 TA Instruments, New Castle, DE, USA). Indium and mercury were used to calibrate the instrument and an empty pan was used as reference. Crumb (4 g, from loaf centre) was compressed with a 2,5 Kg weight to obtain a flat and compact crumb sample to maximize heat transfer within the DSC cell during the experiment. Compressed crumb samples (5-10 mg) were taken and placed in stainless steal pans (Perkin Elmer, USA) that were then hermetically sealed, quench cooled to -80°C and then heated to 130°C at 5°C/min. DSC thermograms were analyzed using a Universal Analysis Software, Version 3.9A (TA Instruments, New Castle, DE). The following parameters were obtained:

"Frozen" water (at the given experimental conditions; FW) was calculated from the endothermic peak around 0°C (ice melting) using the following equation:

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

FW = Frozen water [%]

Enthalpy Ice Fusion [J / g product]

Latent heat of ice fusion = 334 J / g ice

MC = Moisture Content [% g water/ g product].

Retrograded amylopectin was measured by integration of the endothermic peak in the 50-80°C temperature range.

# 3. Water state

## 1.3.1 Water activity

Water activity of bread crumb and crust was measured at 25°C with an Aqualab 4TE (Decagon Devices, Inc. WA, USA). Bread crumb (from loaf centre) or crust samples were broken into small pieces immediately before water activity measurement. At least triplicate samples of crumb and crust were analysed for each bread loaf.

## 1.3.2 Moisture content

Moisture content of crumb (from loaf centre) and crust were determined in triplicate for each bread loaf by weight loss at 105°C (ISCO NSV 9035, ISCO, Milan, Italy) to constant weight.

1.3.3 Molecular mobility - Proton Magnetic Resonance

A low resolution (20 MHz) $^{1}H$ NMR spectrometer (the MiniSpec, Bruker Biospin, Milano, Italy) operating at 25°C was used to study proton molecular mobility by measuring the free induction decay (FID), transverse ($T_2$) and longitudinal ($T_1$) relaxation times and self diffusion coefficient (D). Three g of compressed bread crumb (10 mm high, extracted from loaf centre) were placed into a 10 mm NMR tube that was then sealed with Parafilm® to prevent moisture loss during the NMR experiment. FIDs were acquired using a single 90° pulse, followed by dead time of 7 $\mu$s and a recycle delay of 0.6 s. $T_2$ (transverse relaxation times) were obtained with a CPMG pulse sequence (Carr and Purcell, 1954; Meiboom and Gill, 1958) with a recycle delay of 0.6 s (≥5 T1), an interpulse spacing of 0.04 ms and preselected data points depending on sample. $T_1$ (longitudinal lattice relaxation times) were determined by the inversion recovery pulse sequence with an interpulse spacing ranging from 1 ms to 2500 ms depending on the sample relaxation time, a recycle delay of 0.6 s (≥ 5 $T_1$) and 20 data points. $T_2$ and $T_1$ curves were analyzed as quasi-continuous distributions of relaxation times using a UPEN software (Borgia, Brown and Fantazzini, 1998; Borgia, Brown and Fantazzini, 2000). The proton self diffusion coefficient (D) was obtained with a pulsed-field gradient spin echo (PFGSE) pulse sequence (Stejskal and Tanner, 1965); the Minispec was calibrated with acetic acid (self diffusion coefficient = 1.08 $10^{-9}$ $m^2/s$ at 25°C).

# Section A:

# EFFECT OF MIXING ON BREAD STALING

# THE EFFECT OF AN INNOVATIVE DOUGH MIXER ON BREAD PROPERTIES AND STALING

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## 1. Abstract

The effect of an innovative mixing process that forms a dough almost instantaneously (by subjecting ingredients to a centrifugal force and allowing them to come in contact into a chamber) was evaluated on physico-chemical properties, water dynamics and staling of white bread.

Bread produced with the innovative mixer (IB) was characterized for water activity, moisture content, thermal properties and $^{1}H$ NMR mobility and compared to a control during storage. The water status in the IB product was significantly affected by the innovative mixing process as evidenced a lower proton self diffusion coefficient (D), lower ice melting temperature, lower amount of water extractable by oven drying, minor mobility loss of the more rigid proton fraction ($^{1}H$ FID decay) and larger amount of "exchanging" protons. It is hypothesized that the innovative mixer may cause a stronger solid-liquid interaction in the bread matrix favouring retention of plasticization of the amorphous phase during storage.

## 2. Introduction

Mixing in the production of bread is generally carried out discontinuously by introducing the ingredients (water, flour and others) in a chamber where a rotating shaft provides mechanical energy leading to the formation of a bread dough. Multiple physico-chemical changes take place during the mixing process (generally 10-12 minutes long), including the hydration of solid components, the formation of the gluten network where starch granules

become entrapped, the incorporation of air bubbles, resulting in the formation of a complex visco-elastic matrix (Hibberd and Parker, 1975). An innovative mixer, named Bakmix®, has recently been designed and it was previously described (Storci, Patent DE102005025016, 2005; Curti, Vittadini, Di Pasquale, Riviera, Antoniazzi and Storci, 2010, in press). Briefly, this mixer (Bakmix®, Figure A - 1) provides the simultaneous introduction of dry (stored in [2], volumetrically dosed [3]) and liquid (dosed with a pump and delivered through [4]) ingredients in a chamber [5] containing a fast stirring mechanism. Dry and liquid ingredients are subjected to a centrifugal force that causes their dispersion in air as dust and aerosol, respectively. The dispersed materials come in touch in the chamber [5] inducing an uniform hydration of the surface of each individual dry particle and forming an incoherent matter (1-3 seconds). This is immediately extracted from the chamber and led to into a twin-screw "low pressure extruder" [7] (10-15 seconds, 5 atm and 100 rpm) that favours the transformation of the incoherent wet mass in a "dough" that can undergo normal bread processing procedure.

The described Bakmix® mixing process differs significantly from the traditional mixer in respect to the much higher speed of the process, the very low (almost null) shear applied to the forming dough and the mode of interaction between dry and liquid ingredients. Water is known to play an important role in bread stability: a macroscopic migration of water between crumb and crust due to the presence of a moisture gradient contributes to crumb hardening (Lin and Lineback, 1990) and, therefore, promotes staling (Schiraldi and Fessas, 2001; Baik and Chinachoti, 2002). A change in the microdistribution of water among bread components must also be taken into consideration. It has been reported that this moisture migration causes a decrease in freezable water content (Slade and Levine 1991; Vodovotz, Hallberg and Chinachoti, 1996; Vittadini and Vodovotz, 2003) and water molecules are incorporated in the crystal structure of amylopectin (Imberty and Perez 1988); moreover water migration from gluten to starch and hydrogen bonding between gelatinized (partially pasted) starch granules and the gluten network in bread may be responsible of bread staling, causing a loss of plasticization of the gluten network inducing, consequently, a loss of elasticity of bread crumb (Leung, 1981; Martin, Zeleznak and Hoseney, 1991; Gray and Bemiller, 2003).

Preliminary results on the properties of white bread produced using the Bakmix® indicated that the bread was comparable in appearance and volume to a control bread (whose dough was produced using a traditional mixer; Curti et al., 2010, in press). The Bakmix® bread was found to have a comparable hardness (~120g) to the control in the fresh

products; both breads underwent a similar hardening process during the first three days of storage (up to ~ 350 g), while the Bakmix® bread remained softer than the control at longer storage times (≥ 5 days; Curti et al., 2010, in press). The lower hardness of Bakmix® bread was not attributed to different degrees of recrystallized amylopectin (comparable in the two breads) but it was tentatively attributed to a better plasticization of the solids due to stronger water-solids interactions in Bakmix® (Curti et al., in press). It is hypothesized that the Bakmix® mixing process may lead to the formation of a dough that might favour bread stability because of a different water-solids interaction.

The objective of this work was, therefore, to extensively investigate the effect of the Bakmix® mixing process on the state of water (water activity, moisture content, frozen water content and $^{1}H$ NMR mobility) in white bread during storage.

## 3. Materials and methods

### 3.1 Bread formulation and processing

Bread production was performed according to the official method of American Association of Cereal Chemistry (AACC Method 10-10A) using the following formulation expressed on a flour basis: wheat flour (100), water (65 or 70 or 75) sugar (6) yeast (3), sunflower oil (3), and salt (2). The dough was subjected to resting (1 hour, room temperature), lamination (three times to 3 cm thickness; Kemplex SFP, Kemplex S.N.C, Italy), resting (25 minutes, room temperature), lamination (35 times to 2,5 cm thickness, Kemplex SFP, Kemplex S.N.C, Italy), and was then allowed to rise (50 minutes, 30°C, 85% relative humidity, D-97450, Michael Wenz, Miwe Condo Arnstein, Germany). Baking was carried out at 240°C for 25 minutes in a forced convection oven (D-97450, Michael Wenz, Miwe Condo Arnstein, Germany).

Mixing of the ingredients was performed using either a standard mixer (Kitchen Aid® 5KSM5, Kitchen Aid Europe, Ink. Brussels, Belgium) and the product was named standard bread [**SB**] or the Bakmix® mixer (Storci SPA, Collecchio PR, Italy) and the product was named innovative bread [**IB**]). The acronyms SB and IB are intended for the formulation with a flour: water ratio = 100:65 while the suffixes "70" or "75" were added to SB and IB for the formulations containing higher amount of water (70 and 75, respectively).

At least three bread productions were carried out for each bread formulation and mixer. Breads loaves were allowed to cool to room temperature for two hours prior to be placed in sealed polyethylene bags and then stored at 25°C for 7 days. Bread loaves were analysed

at 0, 1, 3, 5 and 7 days after production. Two bread loaves were sacrificed at each day of analysis.

### 3.2 Bread Characterization

Bread loaves were characterized for macroscopic (volume, hardness), macromolecular (frozen water content, recrystallization of amyolpectin) and water properties (water activity, moisture content and molecular mobility) as reported in the section Methods of analysis.

### 3.3 Statistical analysis

Significant changes ($p<0.05$) of considered properties were evaluated during storage for each bread type (SB and IB) with analysis of variance (ANOVA). An independent student's t-test analysis was used to identify differences between breads produced with different mixers at the same storage time (SPSS v.15, SPSS Inc. IL, USA).

## 4. Results and discussion

The production of bread whose dough was made with the traditional mixer could be carried out only with the formulation containing the lower amount of water (e.g. 65) considered in this study since an unworkable, sticky dough was obtained when using a larger amount of water (e.g. 70 and 75). On the contrary, when a Bakmix® mixer was used, it was possible to produce a dough with a consistency suitable to undergo the bread making process at the three water levels considered (65, 70 and 75).

Bread obtained with the innovative mixer (905 ± 50 ml) had an overall appearance and loaf volume comparable to that of the product that underwent the traditional mixing process (938 ± 38 ml). A higher water content in the formulation caused a slight decrease in the loaf volume to 881 ± 60 ml in IB-70 and a significant reduction to 813 ± 40 ml in IB-75. Also the crumb structure was comparable in SB, IB, IB-70 and IB-75 as verified by the analysis of pores size distribution in the crumb (data not shown, analysis carried out following Chiavaro, Vittadini, Musci, Bianchi and Curti, 2008). IB was found to be softer than SB at longer storage times (≥ 5 days) and the reported different textural attribute could not be ascribable to a reduced amount of recrystallized amylopectin (Curti et al., in press). The higher softness of bread crumb produced with the Bakmix® was even more pronounced in IB-70 and IB-75 up to 14 days of storage (data not shown). A few

indications that a different water status could be found in SB and IB were reported (Curti et al., in press) and, therefore, a thorough characterization of water properties in the two products was carried out.

All bread loaves object of this study (SB, IB, IB-70 and IB-75) were found to lose a comparable amount of water (about 20% of the dough weight) during baking.

Crust of both fresh breads had a moisture content (water extractable at 105°C) of ~ 17 % (g $H_2O$ / 100 g sample) and in the crumb it was found to be ~ 40 % (g $H_2O$ / 100 g sample; Figure A - 2A). During storage bread crumb moisture content decreased to ~ 36 % while the crust moisture content increased to ~ 25 % (Figure A - 2B), as expected, because of macroscopic moisture migration from the wetter bread crumb to the drier bread crust (Kulp and Ponte, 1981; Baik and Chinachoti, 2001). It is noteworthy that the moisture extractable by oven drying from IB crumb was significantly lower than that from SB for the entire storage, suggesting a possible stronger water-solid interaction in this product. A slightly higher crumb moisture content (about 1-2 % as compared to the IB moisture content at each storage time) was found in IB-70 and IB-75 up to 14 days of storage

Water activity of bread fractions in fresh crumb and crust of both bread types (SB and IB) were found to be ~ 0.964 and ~ 0.816, respectively (Figure A - 2B). During storage crumb water activity decreased to ~ 0.959 and crust water activity increased up to ~ 0.935 in both samples as a consequence of the moisture migration from crumb to crust (Figure A - 2B). Water activity of crust and crumb of IB-70 and IB-75 were comparable to those reported for IB at all storage times (data not shown). Although no significant differences were found between the SB and IB breads, the SB crust and crumb water activity values were slightly higher than those of the corresponding IB fractions, suggesting a possible stronger water-solid interaction in the IB product.

The possible stronger solid-water interaction in the IB bread as compared to the SB product, hypothesized based on the reported slightly different water activity and moisture content values, was also suggested by the analysis of the ice melting transition. The major DSC endothermic peak (Figure A - 3) was attributed mainly to ice melting (Vodovotz et al.,1996; Li, Dickinson and Chinachoti, 1998; Baik and Chinachoti, 2001) in all samples. The ice melting endotherm was skewed towards lower temperatures in IB as compared to SB (Figure A - 3). The ice melting onset temperatures are reported in the insert A in Figure A - 3 and they were found to be significantly lower in IB as compared to SB at storage times ≥ 1 day. The lower $T_{on}$ in IB may indicate the presence of a lower temperature melting ice phase in IB, possibly induced by a stronger water-solute interaction and/or an altered ice

crystal distribution in this sample. This possible stronger water-solid interaction was not detected by the analysis of the amount of water that froze under the experimental conditions. The frozen water content (FW) was comparable between the samples and was found to be, in the fresh products, 39.2 ± 3.2 % and 40.1 ± 2.6 % (g frozen $H_2O$ / 100 g water) in SB and IB, respectively.

Water activity, moisture content and frozen water content data all suggested a possible stronger water-solid interaction but they are parameters that measure averaged and long range water properties (Vittadini, Dickinson and Chinachoti, 2002; Vittadini and Chinachoti, 2003; Vittadini, Clubbs, Shellhammer and Vodovotz, 2004; Vittadini, Dickinson and Chinachoti, 2001). A different perspective of the molecular properties of water was obtained using NMR spectroscopy. Multiple $^1H$ NMR experiments were performed to cover a large range of molecular relaxation events. $^1H$ rotational mobility was studied, for the fastest-relaxing component, with a FID experiment while the slower relaxing proton fractions were characterized in terms of $T_2$ and $T_1$ relaxation times distributions. Translational $^1H$ molecular mobility was quantified in terms of the $^1H$ self diffusion coefficient.

The $^1H$ FID decays of SB and IB (both fresh and stored) are shown in Figure A - 4: the first, fast relaxing portion of the FID decay (<0.08 ms) is indicative of the presence of a very rigid 1H population. The $^1H$ FID decay of fresh SB was only slightly less sharp of the fresh IB. The $^1H$ FID rigid component became progressively more relevant during storage more significantly in SB as compared to IB (Figure A - 4). Similar changes in FID were previously reported for gelatinized waxy maize starch (Farhat, Ottenhof, Marie and de Bezenac, 2003) and bread (Sereno, Hill, Mitchell, Scharf and Farhat, 2007). Such changes were attributed by the authors to a reduced mobility of the bread matrix due to both recrystallizing amylopectin and loss of water from the crumb. The lower amount of rigid protons in IB may play a role in the observed softer texture of the product at 7 days of storage. The $^1H$ FID decay in IB-70 and IB-75 was relevantly less sharp than in IB, both fresh and 14 days-stored samples (data not shown), indicating a higher mobility in these breads.

Proton $T_2$ and $T_1$ relaxation decays were analyzed as quasi-continuous distributions of relaxation times using the UPEN software. The $^1H$ $T_2$ distribution spectra were analyzed for $T_2 \geq 0.089$ ms (2 interpulse spacing + instrument dead time) i.e. no extrapolation of $T_2$ values shorter than the measured point on the CPMG was attempted. A representative $^1H$ $T_2$ distribution curve for white bread is reported in Figure A - 5a: three $^1H$ $T_2$ populations were found in both samples and were named starting from the shorter to the longest

relaxation time A, B and C, respectively. In fresh breads, $T_{2A}$ represented a population of protons characterized by relaxation times in the ~ 0.09 – 4 ms range and peaked at ~ 0.15 ms, the $T_{2B}$ protons relaxed in the ~ 5 - 15 ms range and peaked at ~ 10 ms and $T_{2C}$ protons were characterized by longer relaxation times (peak at ~ 100 ms). During storage (Figure A - 5b) $T_{2B}$ (peak time) decreased significantly from ~10 ms in fresh samples to ~6 ms in 7 days stored breads while the overall peak lineshape did not change during storage in the bread object of this study. A significant narrowing and shifting (towards lower relaxation times) of this peak was previously reported in gelatinized waxy maize starch during storage (Farhat et al., 2003). This further indicated that amylopectin recrystallization is not the sole and main event contributing to bread staling but that other factors (such as gluten and/or water redistribution in the amorphous regions of the sample) may play an important role (Hallberg and Chinacoti, 2002; Vodovotz, Vittadini and Sachlebern 2002). $T_{2C}$ (peak temperature, Figure A - 5b) decreased from ~105 ms in fresh samples to ~91 ms during the same length of storage, standing for a decrease of overall proton mobility as previously reported (Chen, Long, Ruan and Labuza, 1997). On the contrary, $T_{2A}$ (peak temperature, Figure A - 5b) did not undergo significant changes during storage. No significant differences were found between the values of $T_{2A}$, $T_{2B}$ and $T_{2C}$ of SB and IB at same storage time. The presence of a higher amount of water in the bread formulation (IB-70 and IB-75) induced a slight shift of $T_{2B}$ towards higher relaxation times from ~9 to 11 ms in fresh samples and from ~ 4 to 6 ms in 14 days stored samples, while $T_{2A}$ and $T_{2C}$ were not affected by the higher amount of water in the formulation (data not shown).

The relative amount of protons in each population was obtained (by UPEN analysis, Borgia et al. 1998, Borgia et al. 2000) and the results were summarized in Figure A - 5c. $T_{2B}$ was the most represented $^1H$ population encompassing ~ 66-67% of the total protons (fresh products), followed by $T_{2A}$ (~29%) while $T_{2C}$ was the smallest $^1H$ population (< 4-5% of total protons). The relative amount of protons in each population changed significantly during storage. $T_{2A}$ (% total protons) decreased significantly from ~29 to ~22% (total protons) both in SB and IB while $T_{2B}$ increased significantly from ~67 to ~73% (% total protons) in both samples. On the contrary, the amount of protons of $T_{2C}$ remained essentially constant during storage in both samples but $T_{2C}$ of IB was slightly, but significantly, larger (of about 1 %) than $T_{2C}$ of SB (Figure A- 5c). The presence of a higher amount of water in the bread formulation (IB-70 and IB-75) did not significantly affect the percentage of protons in the three populations as compared to IB and their changes during storage well reflected those reported for the IB bread.

Multiple $^1H$ $T_2$ populations have been previously reported in baked products by several researchers. Our results are consistent with some previous studies: Engelsen, Jensen, Pedersen, Norgaard and Munck (2001) found three proton $T_2$ populations peaking at ~0.5 ms, ~9-10 ms and ~21-30 ms that were attributed to water associated to protein, water associated to gelatinized starch (and pentosans) and diffusive exchange water between starch and protein, respectively. Wang, Choi and Kerr (2004) studied some model systems (starch gels, gluten gels and starch-gluten gels) and also bread samples to evaluate the effect of moisture content and gluten on their proton mobility. They found two proton populations, peaking at ~0.1 ms and ~3.0 ms and attributed this last population to water associated with starch. Sereno et al. (2007) found one $^1H$ $T_2$ population peaking at ~9 ms representative of the fast proton exchange between water and starch and the restricted water mobility within the polymers matrix. Chen et al. (1997) found three proton populations, peaking at 8-12 $\mu s$, 280-320 $\mu s$ and 2.0-2.6 ms respectively and they attributed the shortest $T_2$ component to water associated to starch and gluten by hydrogen bonding. Also Ruan, Almaer, Huang, Perkins, Chen and Fulcher (1996) observed the presence of two proton populations in sweet rolls, peaking in the microseconds range and a second one peaking in the milliseconds range.

The three proton populations observed in SB and IB are, therefore, tentatively assigned to protons associated to protein (population A), to protons associated with the gelatinized starch phase (population B) and more mobile, exchanging protons (population C). The decrease of the amount of protons belonging to population A and the corresponding increase of the protons in population B might indicate a migration of water from gluten to the starch. The slight, but significantly larger $^1H$ $T_{2C}$ population in the breads produced with the Bakmix process, may indicate the presence of a larger pool of "exchangeable" water that may might provide "flexibility" and "plasticity" to the crumb matrix.

$^1H$ $T_1$ distributions were unimodal and peaked at ~ 90 ms in fresh SB and IB (Figure A - 6). As observed for the 1H $T_{2,}$ also the $^1H$ $T_1$ distribution slightly shifted towards shorter relaxation times during storage, suggesting a decreased overall proton mobility. $^1H$ $T_1$ decreased significantly from ~ 90 to ~ 74 ms during 7 days of storage in both samples. The $^1H$ $T_1$ distribution lineshape was found to be slightly narrower in IB during all storage as compared to SB, possibly suggesting a more homogenous structure in the IB bread.

The self diffusion coefficient (D) could be obtained only until 3 days of storage in both samples due to the fact that at longer storage times it fell below the experimental limit. D was found to be 0.27 ± 0.03 * $10^{-9}$ $m^2/s$ in fresh IB, significantly lower than in SB (D= 0.33

± 0.03 * $10^{-9}$ $m^2/s$), indicating a significant lower $^1H$ translational mobility in the innovative bread, that was retained during storage.

## 5. Conclusions

The effect of the innovative mixing process on the state of water was extensively investigated and evidenced the presence of some small, but significant, differences in the water status between the SB and IB products. In particular, a stronger solid-liquid interaction could be hypothesized in IB as compared to SB based on some experimental evidences: a significantly lower translational transverse proton molecular mobility (D), a lower temperatures ice melting transition, and a lower amount of water extractable by oven drying. Moreover, the IB product was found to undergo minor mobility loss of the more rigid proton fraction ($^1H$ FID decay) during storage, possibly because of the presence of a slightly larger higher mobility population of exchanging protons ($T_{2C}$), that might favour retention of plasticization of the amorphous phase.

## 6. List of Figures

**Figure A - 1**: Schematic representation of the Bakmix® mixer.
[1] motor, [2] flour feeding, [3] volumetric flour doser, [4], inlet of liquid ingredients, [5] mixing chamber, [6] outlet, [7] twin-screw, [8] outlet die. (Curti et al., in press)

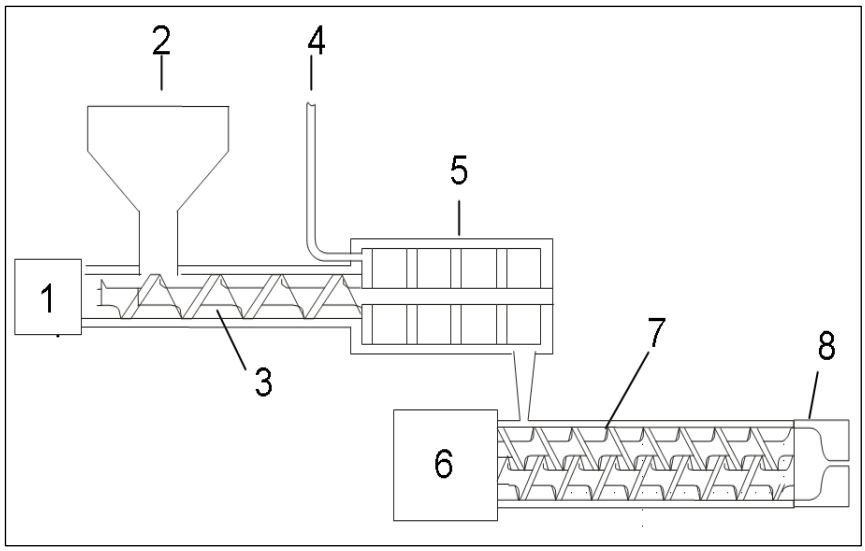


**Figure A - 2**: Moisture content (A) and water activity (B) of crust (open symbols) and crumb (solid symbols) of SB (squares) and IB (circles) during storage.

Error bars represent ±1 standard deviation (n = 9). Mean significant differences for each sample and each location during storage are shown. Symbols (of same shape and colour) with the same letters are not significantly different ($p \leq 0.05$). Small letters were used for SB and capital letters for IB. An asterisk (*) above the symbols indicates significant differences between SB and IB at the same storage time ($p < 0.05$) for the same location.

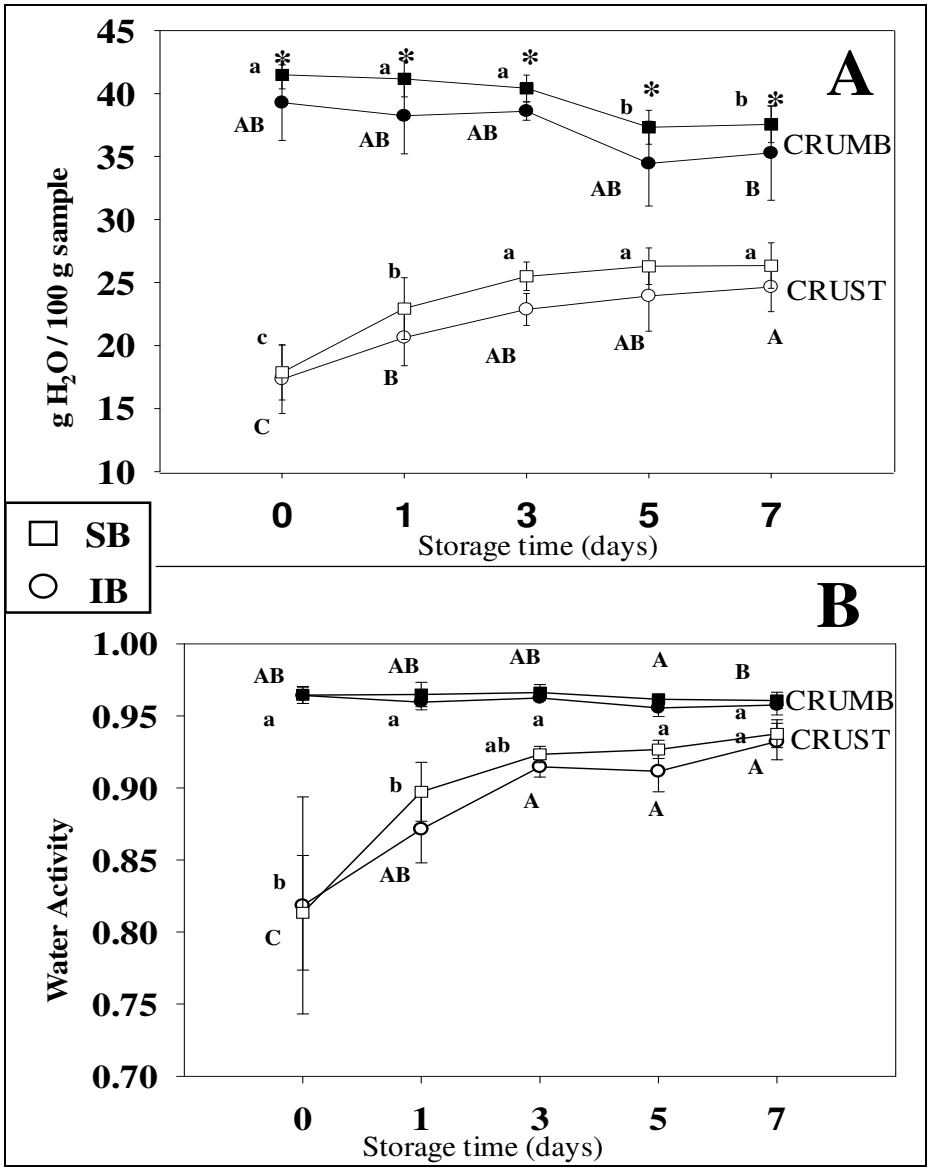
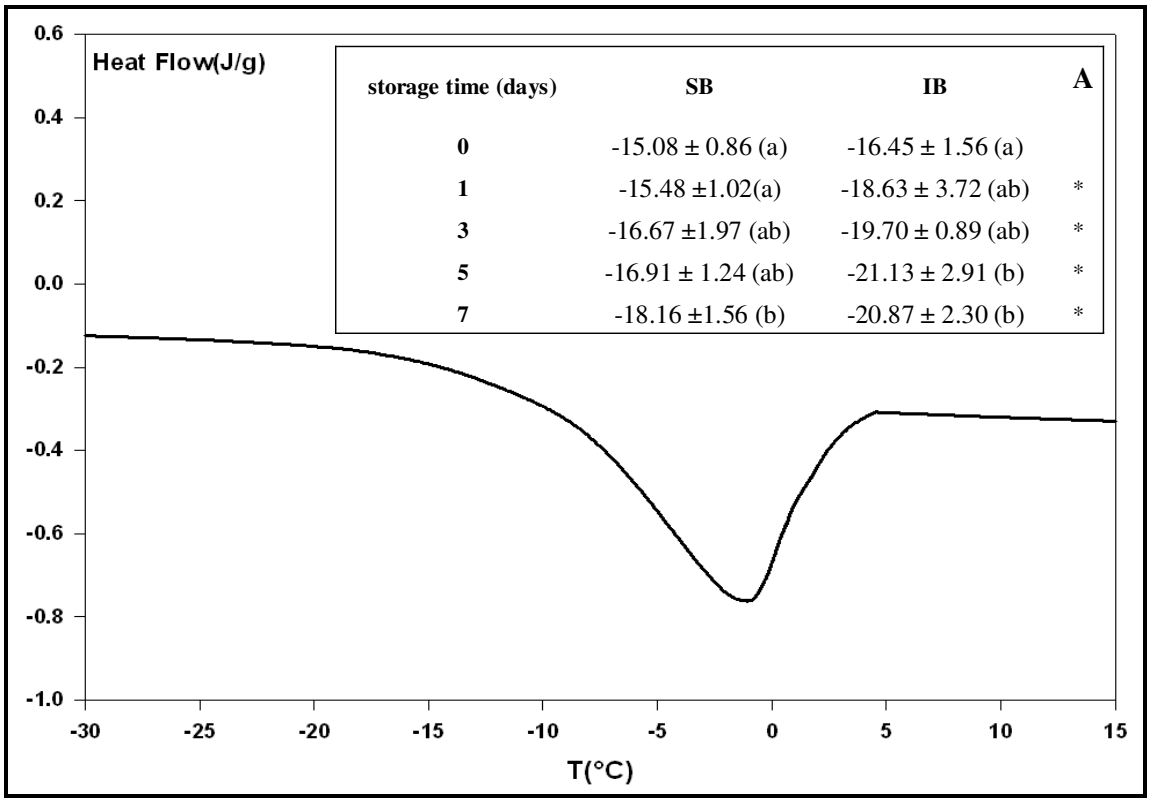


**Figure A - 3**: Typical DSC thermogram of SB and IB in the -30 – 15°C range. $T_{ons}$ for SB and IB for the ice melting transition are reported in insert A.




A

| storage time (days) | SB | IB | |
|---|---|---|---|
| 0 | -15.08 ± 0.86 (a) | -16.45 ± 1.56 (a) | |
| 1 | -15.48 ±1.02(a) | -18.63 ± 3.72 (ab) | * |
| 3 | -16.67 ±1.97 (ab) | -19.70 ± 0.89 (ab) | * |
| 5 | -16.91 ± 1.24 (ab) | -21.13 ± 2.91 (b) | * |
| 7 | -18.16 ±1.56 (b) | -20.87 ± 2.30 (b) | * |

**Figure A - 4**: $^{1}H$ FID of SB and IB during storage

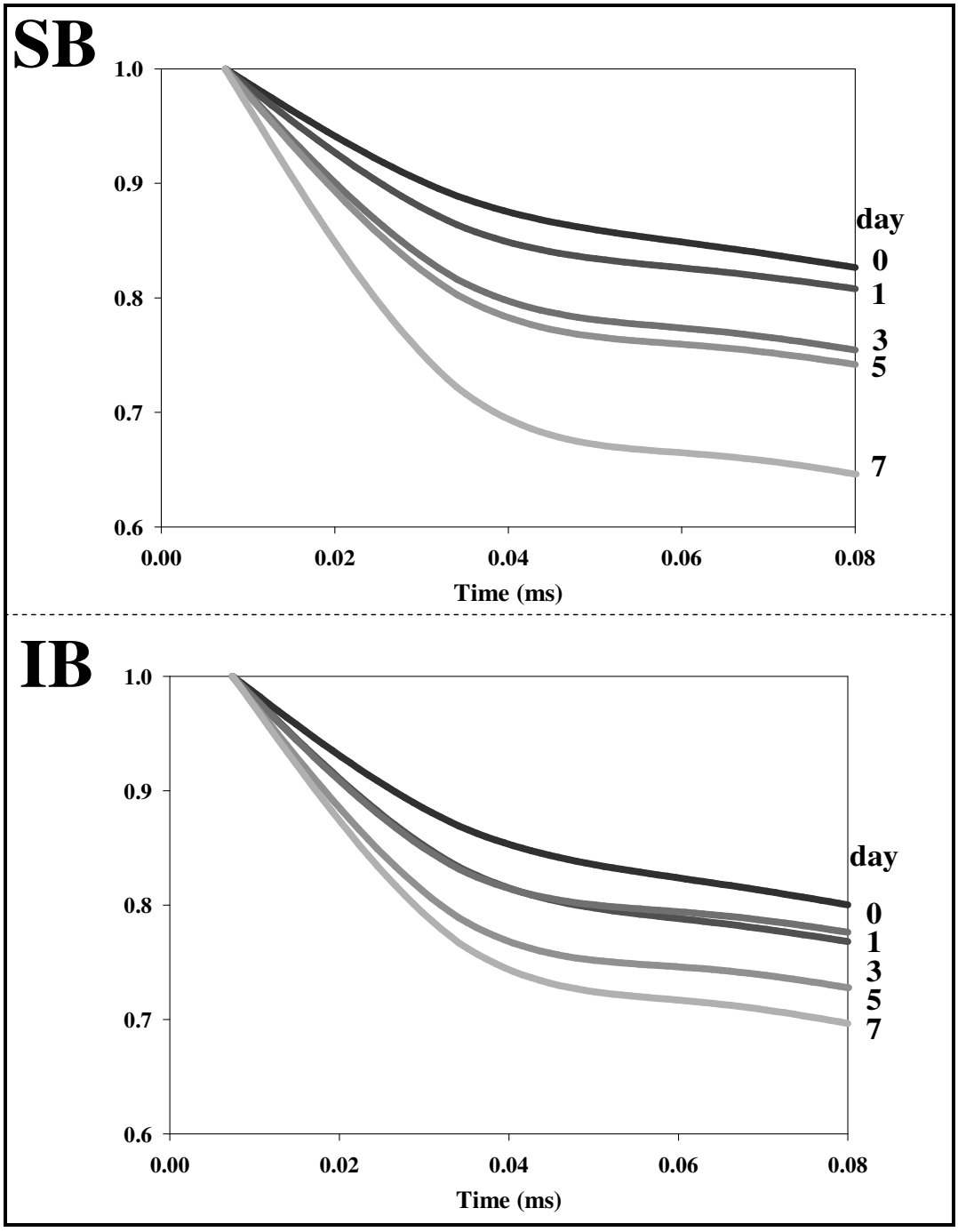


**Figure A - 5**: **a**) – Representative $^1H$ $T_2$ distribution of relaxation times obtained by UPEN software;
**b**) – $^1H$ $T_2$ relaxation times (peaks' times) for SB (black) and IB (White) upon storage;
**c**) - $^1H$ $T_2$ population A, B and C for SB (black) and IB (White)
An asterisk (*) above plot symbols indicates significant differences between SB and IB in the same bread location at the same storage time ($p < 0.05$)

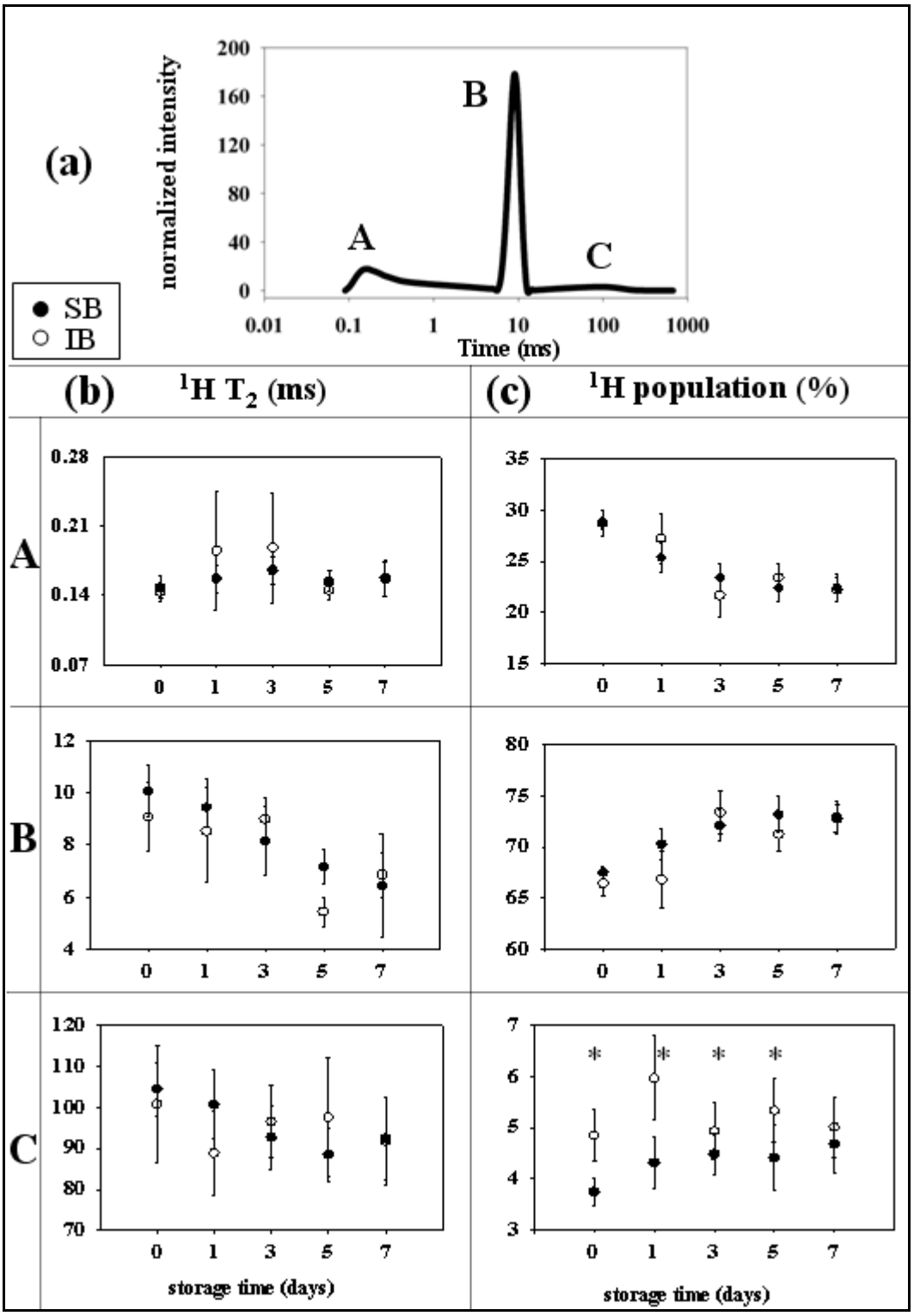
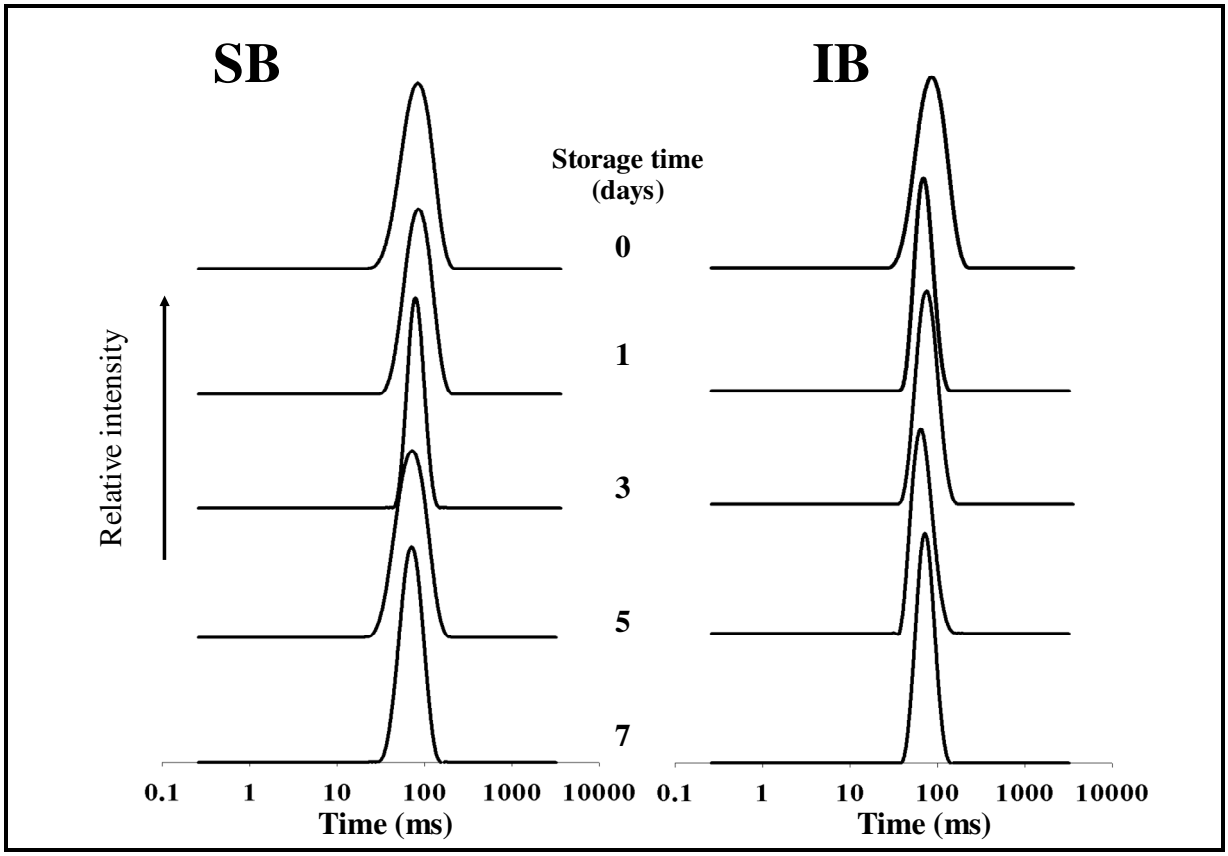


**Figure A - 6**: $^{1}H$ $T_{1A}$ distribution upon storage for SB (left) and IB (right).



# Section B:

# EFFECT OF FORMULATION ON BREAD STALING

# Section B 1

## EFFECT OF THE ADDITION OF BRAN FRACTIONS ON BREAD PROPERTIES AND STALING

Elena Curti, Eleonora Carini, Elena Vittadini, Greta Bonacini

## 1. Abstract

High fibre bread loaves (6.5 g fibre/ 100 g final product) were produced with the addition of wheat durum bran fractions of different compositions and different particle sizes. The effects of the addition of bran fractions on bread properties and staling were evaluated at different levels for macroscopic properties (volume, crumb structure, texture), macromolecular properties (amylopectin recrystallization) and water status (water activity, moisture content, frozen water, $^1$H FID, $^1$H $T_2$ and $T_1$ relaxation time and self diffusion coefficient D) in the fresh products and after 7 days of storage. Macroscopic properties (crust colour, crumb porosity, texture properties) were strongly affected by composition (C1, C2 and C3) and particle size (T1, T2 and T3) of the bran fractions. Volume was significantly decreased only by bran fractions with different composition ("C" samples). In bran enriched samples crust resulted significantly darker and bread crumb showed a generally higher presence of smaller pores. Higher hardness and lower springiness and cohesivity were observed in bran enriched samples, suggesting that bran fractions might have interfered with the development of the gluten matrix. Water status was strongly affected by the addition of bran: water activity, moisture content and frozen water content (as determined by DSC) in the crumb were generally higher, possibly due to a weaker water-solids interaction induced by the presence of bran.

The different composition and the particle size of bran fractions induced a generally higher molecular mobility (slower $^1$H FID decays, higher $^1$H $T_1$ relaxation times, higher self diffusion coefficient). An additional $^1$H $T_2$ protons population not detectable in the STD sample was observed and tentatively attributed to protons related to water-fibre interactions. The altered water status and dynamics found in the bran enriched samples might be related to the higher hardness of these samples.

## 2. Introduction

Bread is defined as "the product obtained by cooking either partially or totally a properly leavened dough, prepared with dry ingredients (wheat flour), water and yeast, with or without salt" (Law n.508, 4/07/1967 and following modifications). White bread is largely consumed around the world and is a primary source of carbohydrates and it could be a good vehicle to higher fibre intake of the population if properly formulated.

According to the American Association of Cereal Chemists (AACC), dietary fibre is defined as "the edible parts of plants or analogous carbohydrates that are resistant to digestion and absorption in the human small intestine with complete or partial fermentation in the large intestine. Dietary fibre includes polysaccharides, oligosaccharides, lignin, and associated plants substances. Dietary fibre promotes beneficial physiological effects including laxation, and/or blood cholesterol attenuation, and/or blood glucose attenuation".

Dietary fibre can be distinguished as soluble (pectins, gums, mucilage) and insoluble (celluloses, lignins, some hemicellulose). Soluble components have been reported to lower the levels of total cholesterol and low density lipoprotein cholesterol in the serum (Glore, van Treeck, Knehans and Guild, 1994; Callaher, Locket and Gallaher, 1992). Insoluble components can contribute to improve the intestinal functions (Özboy and Köksel, 1996). Nutritionists recommend intakes of 30 g per person per day (Miller Jones, 2004) and the interest of the food industry in create products enriched with fibre is rising fast. According to the health claim reported on the Regulation (EC) n.1924/2006 a bread can be defined as a high fibre product (and therefore able to promote beneficial physiological effects when consumed) if contains at least 6 g of fibre per 100 g. An accessible way to nutritionally enhance food products is add fibre to widely consumed food, e.g. bakery products and bread. Wheat bran is the one of the most used sources of dietary fibre added to bread but it has to be taken into account that the addition of fibre (wheat bran) in the formulation alters the rheological and functional properties of the final product, playing a critical role in its quality and stability, especially when added in the amount required to meet the health claim. The effects of dietary fibre on bread properties are mostly due to its affinity for water, influencing the macromolecular and molecular changes related to water interactions: the competition of fibre for water with other bread phases (e.g. gluten, starch) might promote some staling-related phenomena, such as the dehydration of the gluten network, the recrystallization of the amorphous starch and the molecular redistribution of water

among bread components (Gray and Bemiller, 2003). Some studies reported the reduction of loaf volume, the increase of crumb firmness, the dark crumb appearance, an higher water absorption of the dough, that became also shorter and had a reduced fermentation tolerance as the most marked effects of fibre addition on bread properties (Pomeranz, Shogren, Finney and Bechtel, 1977; Lai, Koseney and Davis, 1989; Gan, Galliard, Ellis, Angold and Vaughan, 1992; Knuckles, Hudson, Chiu and Sayre, 1997). Wang, Rosella and De Barbera (2002) reported that fibres from different origin, such as carob fibre, pea fibre and inuline, had a very different influence on bread properties: carob fibre led to the minor modifications related to either texture and overall acceptability of bread. The particle size of fibre has also been reported to strongly affect volume of bread: de Kock, Taylor and Taylor (1999) reported that small sized wheat bran decreased more significantly bread volume in respect to larger sizes of the same bran.

In this study the effects of the addition of bran fractions (in an amount to meet the high fibre claim) obtained from different degree durum wheat of milling and of bran particle sizes on bread properties and staling were evaluated.

# 3. Materials and methods

## 3.1 Bread formulation and processing

### 3.1.1 Bran fractions

Six bran samples were obtained from a local mill. Three bran samples were obtained by milling durum wheat bran to progressively reduce their particle size and the samples were named T1 (larger particles size), T2 (intermediate particle size), and T3 (smaller particle size), respectively.

Three additional bran samples were obtained by milling from the outer bran layer towards the aleuronic layer to obtain fractions with different composition and then grinded to a particle size comparable to that of T3. The samples were named C1 (more external layer), C2, (intermediate layer) and C3 (more internal layer) were produced.

All bran fractions were characterized for their composition with the following methods: proteins (AOAC 950.36), fibre (AOAC 985.29), water (105°C, constant weight), ashes (AOAC 930.22). All bran fractions were characterized also for their particle size.

### 3.1.2 Bread formulation, production and storage

Bread loaves were produced using the formulations reported in Table B 1 - 1. The control sample (STD) was produced using only wheat flour and the fibre enriched samples were

added different amounts of bran fractions to obtain a total fibre content of 6.5% (g fibre/ 100g product) in the final product.

The water amount added to the formulations was adjusted by measuring the dough consistency by a Brabender Farinograph to obtain 500 BU.

Bread was produced with a home bread-maker (Severin BM3986, Germany) using the "wholemeal" program (3h 30 min). Bread loaves were allowed to cool to room temperature for two hours prior to be placed polyethylene bags. Few drops of ethanol were sprinkled in the bags that were than sealed, and stored at 25°C. Three bread loaves were analyzed both fresh (day 0) and after 7 days of storage.

### 3.2 Bread characterization

Bread loaves were characterized for macroscopic (volume, hardness, springiness, cohesivity, crust colour), macromolecular (frozen water content, recrystallization of amyolpectin) and water properties (water activity, moisture content and molecular mobility) as reported in the section Methods of analysis. Crumb structure was also studied as described in the following paragraph.

#### 3.2.1 Crumb structure

Crumb grain was characterized by enumerating the pores present in five preselected dimensional classes based on their area: class 1 0.009–0.02 $mm^2$; class 2 0.02–0.05 $mm^2$; class 3 0.05- 0.1 $mm^2$; class 4 0.1-1 $mm^2$; class 5 1-5 $mm^2$. The number of pores and the area occupied by each class (expressed as percentage of the total number of pores) was evaluated.

#### 3.2.2 Statistical analysis

Significant changes ($p < 0,05$) of considered properties were evaluated during storage (0 and 7 days) for each samples with an independent student's t-test analysis. Analysis of variance (ANOVA, post hoc tests: HSD of Tukey and LSD ) was used to identify differences among all samples of considered properties at the same storage time (SPSS v.15, SPSS Inc. IL, USA). Stars (*) indicate differences of the same sample at different storage times. Capital letters and small letters indicate significant differences among samples at the same storage time (fresh and stored samples respectively).

# 4. Results and discussion

## 4.1 Bran fractions characterization

All bran fractions composition data for proteins, fibre, water , ashes contents and particle sizes are shown in Table B 1 - 2.

Protein content was related to the depth of milling, as expected, and it was higher (~18% g protein/ 100 g sample) in the more internal bran C3 as compared to C2 (~15% g protein/ 100 g sample) and C1 (~10% g protein/ 100 g sample). On the contrary, total dietary fibre (TDF) was higher in the outer layer bran C1 (~63% g fibre/ 100 g sample) as compared to C2 (~50% g fibre/ 100 g sample) and C1 (~36% g fibre/ 100 g sample).Moisture and ashes contents were comparable among all C samples.

The brans with diverse particle sizes (characterized by the same composition) were obtained by sieving bran on different size sieves and the percentage of bran in each dimensional class is reported in Table B 1 – 2, in particular *higher particle size* (T1: ~67% at >500 μm and ~26% at >850 μm), i*ntermediate particle size* (T2: ~40% at >425 μm and ~18% at >300μm), s*maller particle size* (T3: ~30% at >300 μm, ~27% at >180μm and ~20% at <180μm).

## 4.2 Macroscopic bread properties

### 4.2.1 Loaf volume

The volume of all samples is shown in Figure B 1 - 1. C2 and C3 volumes were significantly lower than STD and C1 that were comparable between each other. The composition of bran fractions in C2 and C3 samples may have altered the development of the gluten network, and, therefore, the volume of the loaf.

T1, T2 and T3 volumes were comparable indicating that the different particle size characteristic of the bran in T1, T2 and T3 samples did not significantly affect volume of bread loaves. Our result are in disagreement with the findings of previous studies: Moder, Finneym, Bruinsmam, Ponte and Bolteet (1984) observed that smaller particle size bran (<200 μm) induced higher volumes of bread loaves while opposite results were found by Kock et al. (1999) that found an higher depression of loaf volume in bread containing the smaller particle sized bran (<750 μm) as compared the larger particle size bran (>1800 μm). However the similarity we found in our samples volume might be due to the restricted range of sizes of the bran used by Moder et al. (1984) and to the different

composition of the bran used by Kock et al. (1999) (that were deliberately obtained by different layer of the bran fraction).

### 4.2.2 Crumb structure

Characteristic images of central slices of bread samples and the dimensional analysis of the pores of each formulation are reported in Figure B 1 - 2. The addition of bran to the standard bread formulation generally induced the development of a product with a crumb structure characterized by a larger presence of smaller pores. In particular, all bran added breads showed an higher percentage of small pores belonging to class 1 (0,009 – 0,002 $mm^2$) than STD.

### 4.2.3 Crust colour

Colour coordinates (L*, a*, b*) and ΔE (referred to STD) of the crust of bran enriched breads are shown in Table B 1 - 3. Addition of bran fractions to the bread formulation significantly altered crust colour, as indicated by $\Delta E > 3.5$, as previously reported by Pomeranz et al. (1977). In particular, C2 and C3 resulted significantly distinguishable from STD and C1 in terms of lower brightness (L*) and significantly higher redness (a*). All samples were comparable in terms of yellowness (b*). T1, T2 and T3 differed from STD in terms of significantly lower brightness (L*). All samples were comparable in terms of redness (a*). T3 resulted significantly distinguishable from T1 in terms of yellowness (b*) and was comparable to STD and T2.

### 4.2.4 Crumb texture

Crumb texture (hardness, springiness and cohesivity) of C1, C2, C3, T1, T2 and T3 and STD (fresh and stored) are shown in Figure B 1 - 3. Bran addition in bread formulation generally resulted in an altered textural properties of the fresh products and their changes during storage. Higher crumb hardness was observed in all bran added samples. In fresh samples, C2 and C3 were significantly harder than STD. Also T2 resulted significantly harder than STD, that was comparable to T1 and T3.

Hardness was significantly increased in all stored samples and all fibre enriched samples resulted significantly harder than STD. It was also observed that the increase of hardness (expressed as the increase percentage referred to hardness of fresh samples) was more marked for STD (~280%) than in C1, C2 and C3 (~90-110%) and T1, T2 and T3 (~120-160%). Hardening of bread crumb is partially due to the macroscopic migration of water occurring from the wetter crumb to the drier crust and the minor hardening observed in the bran enriched samples might be due to an influence of bran on water-solid interactions and hence on water macroscopic migration.

Crumb springiness and cohesivity were significantly lower in all bran added samples at day 0 and they significantly and similarly decreased during storage in all samples.

### 4.2.5 Water activity

Water activity of crust and crumb of C1, C2, C3, T1, T2 and T3 and STD, fresh and stored, are shown in Figure B 1 – 4A and 4B respectively. Fresh samples showed a lower crust water activity and an higher crumb water activity, as expected. All fresh samples were generally comparable for crust water activity independently of formulation but not for crumb water activity, that was significantly higher in bran enriched samples. As a consequence of the macroscopic migration on water from crumb to crust, driven by the gradient of moisture existing between the two portions of the product, crust water activity increased during storage while crumb water activity decreased.

During storage, crust water activity significantly increased in all samples and it resulted significantly higher in C3, T2 and T1 as compared to STD after 7 days of storage. A significant decrease in crumb water activity was observed only in C1, C2, C3 and C2 had the highest crumb water activity among all samples. Crumb water activity in T1, T2 (and STD) did not significantly decrease during storage but it was still significantly higher in T1, T2 and T3 as compared to STD in 7 days old bread. Moreover it was observed that the decrease of water activity in the crumb (expressed as the increase percentage referred to day 0 values) was more marked for C1, C2 and C3 (~0.84%) than in T1, T2 and T3 (~0.22%).

### 4.2.6 Moisture content

Moisture content of crust and crumb of C1, C2, C3, T1, T2 and T3 and STD (fresh and stored) are shown in Figure B 1 - 5Aand 5B respectively. All fresh samples showed a lower crust moisture content and an higher crumb moisture content, as expected. At day 0, crust moisture contents were comparable among C1, C2, C3 and STD and significantly higher in T1, T2 and T3 as compared to STD. Crumb moisture contents were significantly higher in all bran enriched samples than in STD. This result may be related to a weaker water-solid interaction, possibly induced by the presence of fibre, that favoured moisture extraction in the drying oven.

During storage crust moisture content increased and crumb moisture content decreased, as a consequence of the macroscopic migration of water occurring from the wetter crumb to the drier crust. Crust moisture content significantly increased in all samples and it was significantly higher in all stored bran enriched sample as compared to STD. During storage crumb moisture content significantly decreased in all samples except for T1 and T2 and it

was significantly higher in all the fibre enriched samples. Moreover, it was observed that the increase of moisture content in the crust (expressed as the increase percentage referred to day 0 values) was more marked for all bran enriched samples (from ~40 to 60%) than in STD (~30%). Moisture content of bread crust changed more markedly as compared to the smaller moisture content change of the crumb, probably because only water molecules from the nearest portion of crumb could migrate in the storage period considered. These results may indicate that fibre strongly altered and weakened water-solid interactions, as suggested by the larger increase observed in the crust in the bran enriched samples as compared to STD.

The decrease in the crumb moisture content was generally more marked for C1, C2, C3, T3 and STD (~3%) than for T1, T2 (~1%), suggesting that the larger particle size of T1 and T2 as compared to C1, C2, C3 and T3 (comparable among each other) may have not so strongly altered the water – solid interactions.

### 4.3 Macromolecular bread properties

#### 4.3.1 Thermal analysis

The thermograms of fresh samples exhibited one major endothermic transition around 0°C and, in stored samples, also a second minor endothermic event occurred at higher temperatures (50–80°C), as the samples were heated from −80 to 130°C. The major DSC endothermic peak around 0°C was mainly attributed to ice melting (Vodovotz, Hallberg and Chinachoti, 1996; Li, Dickinson and Chinachoti, 1998; Baik and Chinachoti, 2001) and it was used to calculate the FW of all samples (Figure B 1 - 6).

At day 0 the frozen water content (FW) was significantly higher in all bran enriched samples, except for T1, that was comparable to STD. This result might be associated with the higher moisture content observed in these sample and/or a weaker water-solids interaction induced by the presence of bran. The higher particle size in T1 may have not allowed the bran in the sample to strongly interact with water and hence affected to a less extent water- solids interactions in this sample, resulting in a lower frozen water content.

The decrease of the frozen water content resulting from the addition of non-flour ingredients was previously reported for wheat tortillas: Serventi, Carini, Curti and Vittadini (2008) produced nutritionally enhanced tortillas prototypes by using different ingredients, such as carrot juice, soy flour and wholemeal kamut flour, and they observed that these ingredients strongly altered the water-solids interactions, inducing a significant decrease for

carrot prototype and a significant increase for soy and kamut prototypes in frozen water content.

Frozen water content significantly decreased in all samples (except for T3) resulting from the migration of a portion of water from gelatinized starch towards the more rigid amorphous and crystalline domains that so became unfreezable (Baik and Chinachoti, 2000; Hallberg and Chinachoti, 2002; Ribotta and Bail, 2007; Kerch et al. 2008). C3 showed significantly higher values of FW than STD, C1 and C2, comparable among each other. The significantly higher FW content in C3 might be related to an altered water-solid interaction induced by the higher presence of non-gluten proteins (18.6 %) in C3 bran fraction. FW content was significantly higher in T1 than in T2 and T3 (comparable between each other) as compared to STD at 7 days of storage.

The endothermic peak observed in the thermograms of stored bread crumb over the 50-80°C temperature range was attributed to crystalline amylopectin melting as previously reported (Russell et al. 1983). The enthalpy values of the endothermic peak for all considered stored samples are shown in Figure B 1 - 7. Recrystallized amylopectin was comparable in C1, C2, C3 and STD and it was significantly higher in T1 and T3 as compared to STD. The samples with comparable recrystallized amylopectin were characterized by significantly different hardness values, indicating that amylopectin recrystallization is not the sole and main event contributing to bread firming, as previously reported by others (Hallberg and Chinacoti, 2002; Vodovotz, Vittadini and Sachlebern 2002).

## 4.4 Molecular bread properties

Molecular characterization was carried out with multiple $^{1}H$ NMR experiments to cover a large range of molecular relaxation events. $^{1}H$ rotational mobility was studied, at 20 MHz, for the fastest-relaxing component, with a FID experiment while the slower relaxing proton fractions were characterized in terms of $T_2$ and $T_1$ relaxation times distributions. Translational $^{1}H$ molecular mobility was quantified in terms of the $^{1}H$ self diffusion coefficient. $^{1}H$ FID decays of C1, C2, C3 and STD are shown in Figure B 1 - 8: the first, fast relaxing portion of the FID decay (<0,08 ms) is indicative of the presence of a very rigid $^{1}H$ population. $^{1}H$ FID decays of the bran enriched samples were sharper than STD $^{1}H$ FID at day 0, indicating an higher molecular mobility. The $^{1}H$ FID rigid component became progressively more relevant during storage in all samples, as already reported by other authors for gelatinized waxy maize starch (Farhat, Ottenhof, Marie and de Bezenac, 2003) and bread (Sereno, Hill, Mitchell, Scharf and Farhat, 2007). Such changes were attributed by

the authors to a reduced mobility of the bread matrix due to both recrystallizing amylopectin and loss of water from the crumb. It was observed that C3 underwent a more marked loss in mobility than C1 and C2, comparable between each other, and STD.

$^1$H FID decays of T1, T2, T3 and STD are shown in Figure B 1 - 8. The bran enriched samples were more mobile than STD at day 0. The $^1$H FID rigid component became progressively more relevant during storage in all samples, as reported above. It was also observed that T1 underwent a less marked loss in mobility than T2 and T3 (comparable) and STD respectively.

The $^1$H $T_2$ distributions obtained using an UPEN software were analyzed for $T_2 \geq 0.089$ ms (2 interpulse spacing + instrument dead time) to avoid extrapolation of $T_2$ values at times shorter than the first point measured with the CPMG experiment. $^1$H $T_2$ quasi-continuous distributions of C1, C2, C3 and STD are shown in Figure B 1 - 9. Three $^1$H $T_2$ populations were found in STD and were named starting from the shorter to the longest relaxation time A, B and C, respectively. The relative amount of protons in each $^1$H $T_2$ population was also obtained by the UPEN software. "A" represented a population of protons characterized by relaxation times in the ~ 0.5-1 ms range ($T_{2A}$); the "B" protons relaxed in the ~ 8-10 ms range ($T_{2B}$); "C" protons were characterized by relaxation times longer than 20 ms ($T_{2C}$). Three populations of protons were found also in C1 and they were comparable to STD for $T_2$ relaxations times but not for the relative proton abundance. In fresh samples (STD and C1), population A was significantly higher in STD (~29%) than in C1 (~ 26%) while population B was significantly lower in STD (~66%) than in C1 (~69%). Four $^1$H $T_2$ populations were found in C2 and C3 (fresh samples) and were named A, B, C and D representative of protons relaxing at ~ 0.3-0.8 ms ($T_{2A}$), ~ 3-5 ms ($T_{2B}$), ~ 7-8 ms ($T_{2C}$) and ~ 90-110 ms ($T_{2D}$) respectively. Population A encompassed ~ 8%, population B ~ 14-17%, population C ~ 69-71% and population D ~ 5-6%.

During storage no relevant changes were observed $T_{2A}$ and $T_{2C}$ of STD and C1 while $T_{2B}$ significantly decreased to ~8ms in STD and to ~6ms in C1. The relative amounts of protons in population A and B changed significantly during storage with A decreasing significantly to ~26% in STD and to ~24% in C1 and B significantly increasing to ~71 % in STD and 75% in C1. Population C remained almost constant during storage. In stored C2 e C3, only two populations were found: a broader population named ABC, encompassing ~ 93% (characterized by the same relaxation times reported for the fresh sample) and a smaller population D, that corresponded to population D found in fresh samples.

The presence of multiple $^1H$ $T_2$ populations has been previously reported in baked products by several researchers. Our results are consistent with some previous studies: Engelsen et al. found three proton $T_2$ populations (with a 23.2 MHz spectrometer) peaking at ~0.5 ms, ~9-10 ms and ~21-30 ms that were attributed to water associated to protein, water associated to gelatinized starch (and pentosans) and diffusive exchange water between starch and protein, respectively. Wang, Choi and Kerr (2004) studied (with a 20 MHz spectrometer) some model systems (starch gels, gluten gels and starch-gluten gels) as well as bread samples to evaluate the effect of moisture content and gluten on proton mobility. They found two proton populations, peaking at ~0.1 ms and ~3.0 ms and attributed this last population to water associated with starch. Sereno et al. (2007) found one $^1H$ $T_2$ population peaking at ~9 ms (with a 23 MHz spectrometer) representative of the fast proton exchange between water and starch and the restricted water mobility within the polymers matrix. According to these studies, the three proton populations observed in STD were, therefore, tentatively assigned to protons associated to water-protein phase (population A), to protons associated with the starch phase (population B) and more mobile, exchanging protons (population C). The decrease of the amount of protons belonging to population A and the corresponding increase of the protons in population B might indicate a migration of water from the gluten domain to the starch domain during storage. C1 showed comparable $^1H$ $T_2$ protons distributions to STD except for the significant differences observed between the two samples (fresh and stored) in the abundance of A and B protons populations and therefore $^1H$ populations in C1 were assigned as described above for STD.

The presence of four $T_2$ protons populations in fresh C2 and C3 indicated that the addition of the bran fractions may have affected more strongly molecular mobility in respect to C1, due to the different composition of the bran fractions that affected the water distribution between the gluten matrix and the starch phase. Molecular mobility was found to change relevantly during storage in C2 and C3, showing a unique proton population encompassing 95% resulting from the overlapping of the A, B and C of proton populations of fresh samples that at 7 days of storage were no longer resolved (clearly separated) as the protons underwent exchange within the NMR experimental time-frame.

$^1H$ $T_2$ quasi-continuous distributions STD, T1, T2 and T3 are shown in Figure B 1 - 10. $^1H$ $T_2$ distributions or relaxation times of STD have already been discussed previously. Four $^1H$ $T_2$ populations were found in all the bran enriched samples (fresh samples) and were named A, B, C and D representative of protons relaxing at ~ 0.15-0.7 ms ($T_{2A}$), ~ 1-2 ms ($T_{2B}$), 8.5 ms

($T_{2C}$) and ~ 105 ms ($T_{2D}$) respectively. Population A encompassed ~ 8-9%, population B ~ 16-17%, population C ~ 68-69% and population D ~ 5-6%. Molecular mobility was found to change during storage in bran enriched samples. Seven days old T1 bread crumb showed the presence of three protons populations named A', B' and C' encompassing ~9%, ~85% and ~6% respectively (characterized by four relaxation times as observed in fresh samples, among which only $T_{2C}$ was found to decrease from 8.5 ms to 7 ms). It may be hypothesized that B' resulted from populations B and C observed in fresh T1 samples. T2 e T3 showed a unique proton population encompassing 93-94% resulting from the overlapping of the A, B and C of proton populations of fresh samples that at 7 days of storage were no longer resolved (clearly separated). It may be hypothesized that the additional protons population, observed in fresh and stored bran enriched samples, could be related to bran-water molecules interactions and could have acted as an exchangeable protons pool between the gluten matrix and the starch phase during storage.

Proton $T_1$ distributions of STD, C1, C2, C3 T1, T2 and T3 (data not shown) were unimodal and representative of a single observable proton population. The relaxation times, representative of the major peak of $^1H$ $T_1$ distributions, for fresh and stored samples are shown in Table B 1 - 4. In fresh samples, C1, C2 and C3 showed significantly higher $^1H$ $T_1$ relaxations times than STD while $^1H$ $T_1$ relaxation times were significantly lower in T3 and comparable between STD, T2 and T1.

During storage $^1H$ $T_1$ relaxations times significantly decreased in STD and generally increased in the bran enriched samples. This finding may suggest the presence of a fraction of protons with high molecular mobility in the bran enriched samples that might be related to a fraction of water loosely interacting with solids due to the presence of bran (as suggested by a generally higher frozen water content in the fibre enriched samples).

Self diffusion coefficients D (that quantifies the translational motions of protons) of STD compared to C1, C2, C3 and T1, T2, T3 are shown in Table B 1 - 5. D of bran enriched samples C1, C2, C3 were significantly lower in respect to STD both at day 0 and day 7. Also D values of fresh T1, T2 e T3 were significantly lower in respect to STD, while in stored samples D values were significantly lower in T3 in respect T1 and T2 and comparable to STD. Moreover, it was observed that the self diffusion coefficients of bran enriched samples C1, C2, C3 and T1 significantly increased during storage while STD D values significantly decreased. The D values observed in all bread samples (both fresh and stored) are a few orders of magnitude lower than for pure water at 25°C ($2.29 *10^{-9}$ $m^2*s^{-1}$) as expected, due to the solid-like nature of the bread matrix. Our results agree with those reported by

previous studies on wheat starch gels (~0.5 * $10^{-9}$ $m^2*s^{-1}$ 0.67 g of water/ g of solids at 25°C; Gomi, Fukuoka, Mihori and Watanabe, 1998) and starch-gluten-water mixtures (~0.2-0.6 * $10^{-9}$ $m^2*s^{-1}$ at 0.54 - 1.00 g of water/g of solids at 30°C; Umbach, Davis, Gordon and Callaghan, 1992) but they are not comparable to those found by Baik and Chinachoti (2003) in white bread (0.067 * $10^{-9}$ $m^2*s^{-1}$ at 35°C). These authors also reported a significant decrease of D during storage that was found, in this study, only in the STD sample. It is therefore hypothesized that the significant increase observed in the bran enriched samples might stand for an higher ability of water to diffuse in the bread matrix due to the weaker water-solids interaction of these samples.

## 5. Conclusions

High fibre bread loaves were produced using different types of wheat durum bran fractions. The addition of bran fractions significantly affected bread properties and storage stability.

At a macroscopic level, composition (C1, C2 and C3) of bran fractions strongly affected volume and porosity, inducing a significant decrease of loaves volume and an increase in the smaller pores of the crumb. Particle size (T1, T2 and T3) affected crumb porosity, inducing a higher percentage of smaller pores, but it did not affect the volume of the loaves (at least in the range of sizes considered in this study). These results could be related to bran fractions impeding the proper formation of the gluten network and therefore on its capacity to retain gas.

Bran enriched samples crust resulted significantly darker and strongly distinguishable from the control sample (STD). Texture of samples was strongly affected by composition and particle size of bran fractions. Bran enriched samples were significantly harder and less cohesive and elastic, possibly suggesting that the gluten matrix did not undergo a proper development in these samples.

The composition and the particle size of bran fractions affected water status. Water activity, moisture content and frozen water content (as determined by DSC) in the crumb were generally higher, possibly due to a weaker water-solids interaction induced by the presence of bran. Molecular mobility was relevantly affected by the addition of all considered bran fractions. The different composition and the particle size of bran fractions affected the water distribution among bread components and induced a generally higher molecular mobility (slower $^1H$ FID decays, higher $^1H$ $T_1$ relaxation times, higher self diffusion coefficient) both in fresh and stored samples. $^1H$ $T_2$ distributions of relaxation times in bran enriched samples

were more “heterogeneous”: an additional protons population, not detectable in the STD sample, was found and tentatively attributed to protons related to water-fibre interactions. It may be speculated that some water molecules related to this protons population might not be available for the development/plasticization of the gluten network during bread production. These findings suggest that addition of bran in the bread formulation strongly altered the water distribution and dynamics resulting in a harder product.

## 6. List of Tables

**Table B 1 - 1**: Breads formulation

| Ingredient (%) | STD | C1 | C2 | C3 | T1 | T2 | T3 |
|---|---|---|---|---|---|---|---|
| Wheat flour | 100,0 | 87,0 | 83,0 | 76,5 | 84,0 | 84,0 | 84,0 |
| Bran fraction | / | 13,0 | 17,0 | 23,5 | 16,0 | 16,0 | 16,0 |
| Sugar | 4,0 | 4,0 | 4,0 | 4,0 | 4,0 | 4,0 | 4,0 |
| Salt | 2,0 | 2,0 | 2,0 | 2,0 | 2,0 | 2,0 | 2,0 |
| Yeast | 3,0 | 3,0 | 3,0 | 3,0 | 3,0 | 3,0 | 3,0 |
| water | 55,2 | 59,8 | 59,2 | 61,2 | 63,1 | 62,5 | 63,3 |
| Sunflower seeds oil | 3,0 | 3,0 | 3,0 | 3,0 | 3,0 | 3,0 | 3,0 |

**Table B 1 - 2:** Composition and particle size of bran fractions

| | % sieve weight ($\mu$m) | | | | | | | composition(%) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | >1000 | >850 | >500 | >425 | >300 | >180 | <180 | Proteins | Moisture | Ashes | TDF |
| **C1** | / | / | 0,4 | 7,5 | 29,8 | 33,8 | 28,5 | 9,9 | 11,6 | 6,3 | 63,0 |
| **C2** | / | / | / | 6,4 | 30,4 | 35,6 | 27,6 | 15,0 | 11,7 | 6,3 | 49,0 |
| **C3** | / | / | / | 10,1 | 25,9 | 39,3 | 24,7 | 18,6 | 12,0 | 5,8 | 36,0 |
| **T1** | 4,2 | 26,0 | 67,7 | 2,1 | / | / | / | 13,7 | 12,7 | 5,8 | 52,0 |
| **T2** | / | / | 13,4 | 40,5 | 18,0 | 16,0 | 12,1 | 13,7 | 9,8 | 5,8 | 52,0 |
| **T3** | / | / | / | 21,6 | 30,6 | 27,0 | 20,8 | 13,7 | 8,1 | 5,8 | 52,0 |

**Table B 1 - 3:** Bread crust colour attributes

| | STD | C1 | C2 | C3 |
|---|---|---|---|---|
| **L*** | 72,57±2,78$^{a}$ | 71,40±1,89$^{a}$ | 68,03±2,45$^{b}$ | 62,27±2,43$^{c}$ |
| **a*** | 3,92±1,94$^{b}$ | 3,37±0,76$^{b}$ | 5,79±0,99$^{a}$ | 7,08±2,43$^{a}$ |
| **b*** | 20,67±1,95$^{a}$ | 20,19±1,39$^{a}$ | 21,66±1,10$^{a}$ | 21,05±1,10$^{a}$ |
| **ΔE** | / | 3,56±2,52 | 15,98±13,21 | 61,82±29,47 |
| | **STD** | **T1** | **T2** | **T3** |
| **L*** | 72,57±2,78a | 68,06±2,24$^{b}$ | 68,03±2,28$^{b}$ | 68,62±1,90$^{b}$ |
| **a*** | 3,92±1,94b | 4,57±1,58$^{a}$ | 4,74±1,37$^{a}$ | 5,01±1,61$^{a}$ |
| **b*** | 20,67±1,95a | 19,15±2,47$^{bc}$ | 19,29±1,93$^{ac}$ | 20,75±1,32$^{a}$ |
| **ΔE** | / | 17,86±10,76 | 16,58±11,69 | 11,48±8,36 |

**Table B 1 - 4**: $^{1}$H $T_1$ relaxation times (peak) of fresh and stored STD, C1, C2, C3, T1, T2 and T3

| $^{1}$H $T_1$ (ms) | Day 0 | Day 7 | |
|---|---|---|---|
| **STD** | 89.94$^{c}$± 4.21 | 85.47$^{d}$ ± 3.28 | * |
| **C1** | 96.99$^{b}$ ± 1.72 | 99.60$^{c}$ ± 1.61 | * |
| **C2** | 95.09$^{b}$ ± 0.86 | 103.15$^{b}$ ± 3.95 | * |
| **C3** | 100.40$^{a}$ ± 1.15 | 108.15$^{a}$ ± 4.86 | * |
| **T1** | 90.21$^{ab}$ ± 5.28 | 97.54$^{a}$ ±0.72 | * |
| **T2** | 95.11$^{ab}$ ±8.40 | 93.88$^{a}$ ± 0.71 | |
| **T3** | 95.87$^{a}$ ±2.15 | 95.41$^{a}$ ±4.08 | |

**Table B 1 - 5**: $^{1}H$ self diffusion coefficients (D) of fresh and stored STD, C1, C2 C3, T1, T2 and T3

| D (*$10^{-9}$ $m^2$*$s^{-1}$) | Day 0 | Day 7 | |
|---|---|---|---|
| STD | $0.401^{a} \pm 0.024$ | $0.442^{a} \pm 0.019$ | * |
| C1 | $0.379^{b} \pm 0.023$ | $0.414^{b} \pm 0.030$ | * |
| C2 | $0.375^{b} \pm 0.018$ | $0.380^{b} \pm 0.029$ | * |
| C3 | $0.365^{b} \pm 0.023$ | $0.390^{bc} \pm 0.033$ | * |
| T1 | $0.415^{b} \pm 0.021$ | $0.461^{a} \pm 0.032$ | * |
| T2 | $0.411^{b} \pm 0.017$ | $0.459^{a} \pm 0.015$ | * |
| T3 | $0.446^{a} \pm 0.018$ | $0.426^{b} \pm 0.027$ | * |

## 7. List of figures

**Figure B 1 - 1:** Volumes of samples C1, C2, C3 (left) and T1, T2, T3 (right) compared to STD

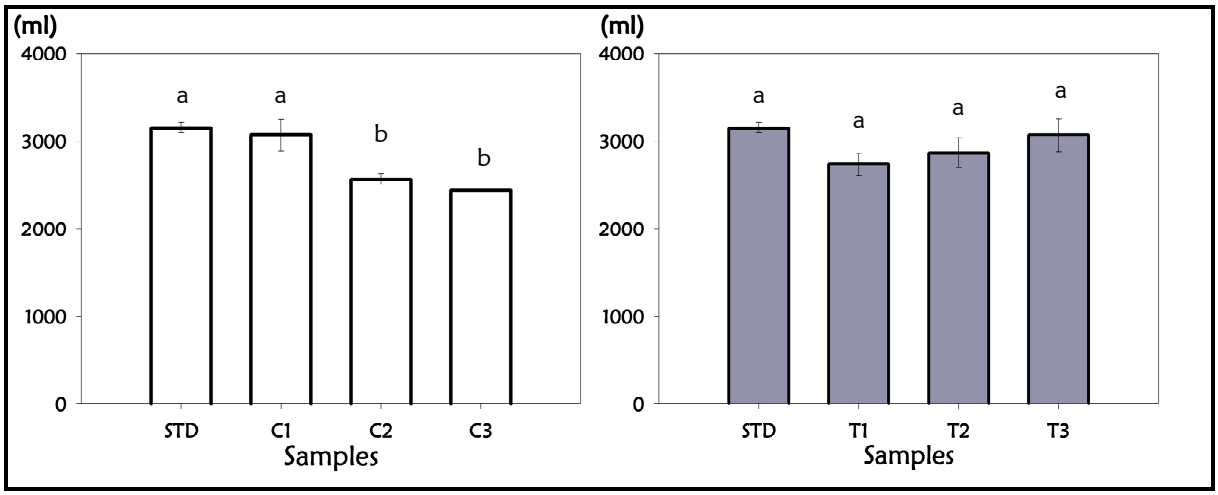


**Figure B 1 - 2:** **A** Percentage (%) of total pores in 5 classes of area* of STD, C1, C2, C3

**B :** Percentage (%) of total in 5 classes of area* of STD, T1, T2, T3

** (class 1 0.009–0.02 mm²; class 2 0.02–0.05 mm²; class 3 0.05- 0.1 mm²; class 4 0.1-1 mm²; class 5 1-5 mm²)*

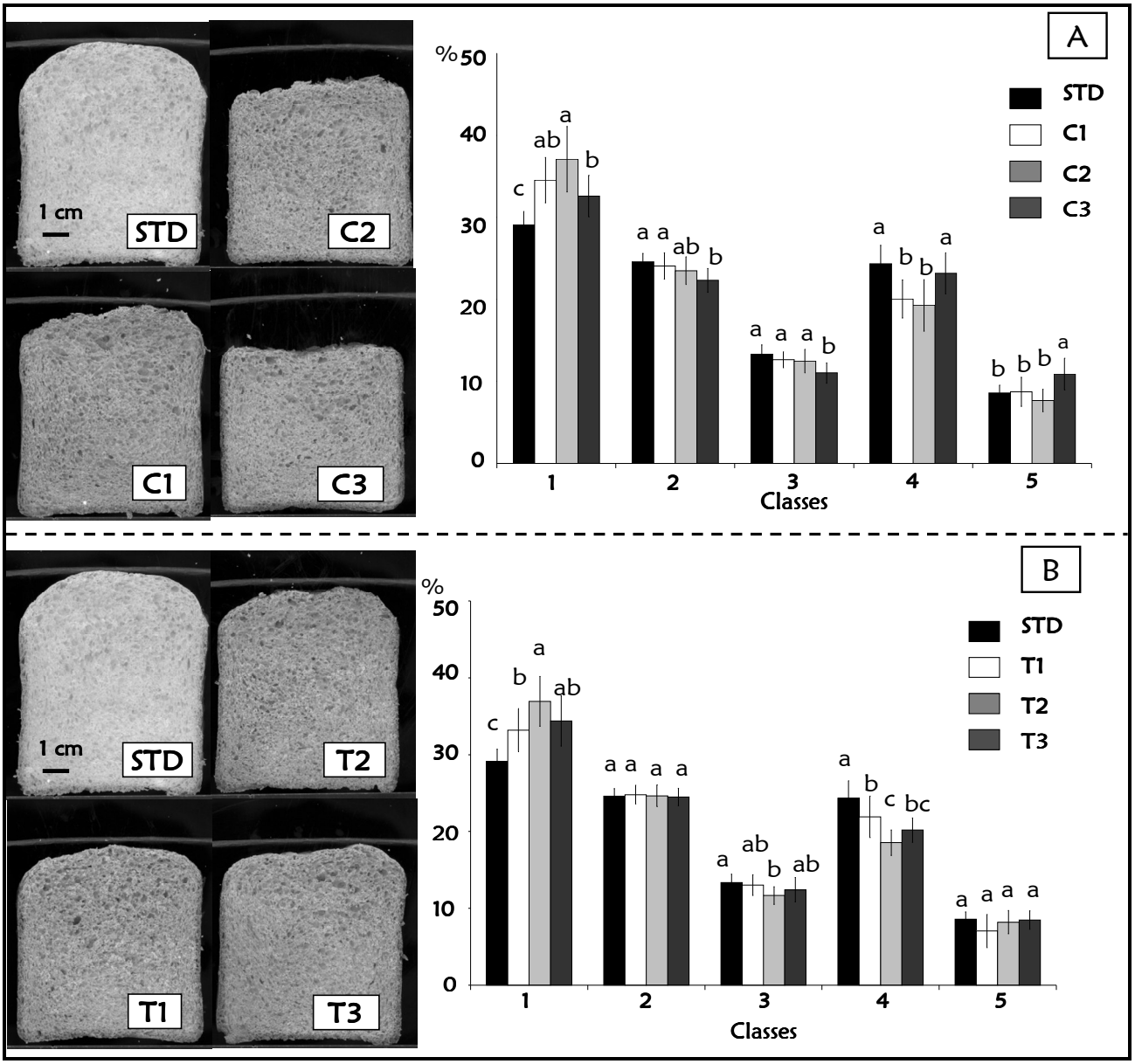


**Figure B 1 - 3:**

**A-** Crumb hardness of fresh and stored STD, C1, C2, C3 (left) and STD, T1, T2, T3 (left)
**B-** Crumb springiness of fresh and stored STD, C1, C2, C3 (left) and STD, T1, T2, T3 (left)
**C-** Crumb cohesivity of fresh and stored STD, C1, C2, C3 (left) and STD, T1, T2, T3 (left)

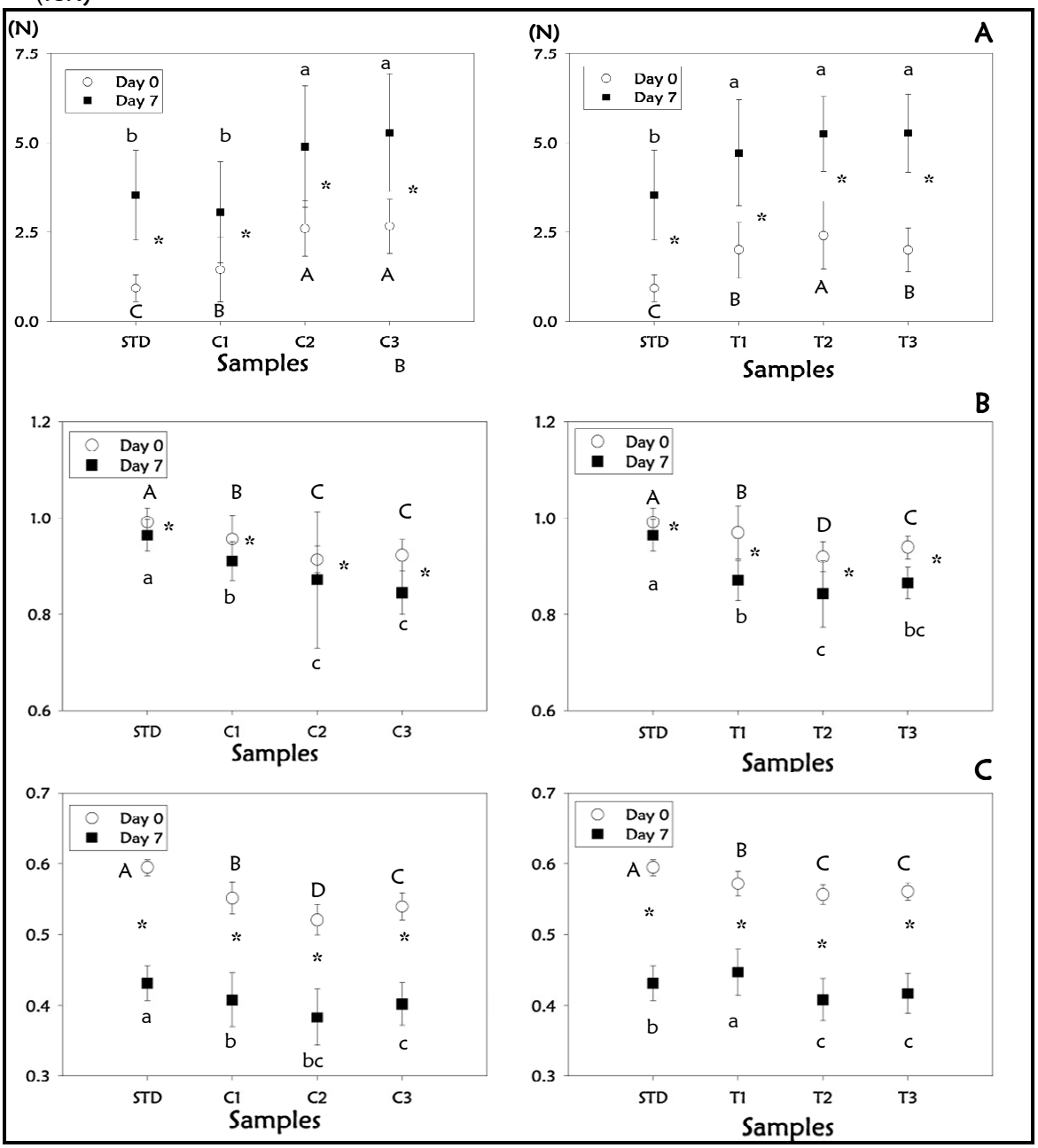


**Figure B 1 - 4:**

**A -** Crust (left) and crumb (right) water activity of fresh and stored STD, C1, C2, C3

**B:** Crust (left) and crumb (right) $a_w$ of fresh and stored STD, T1, T2, T3

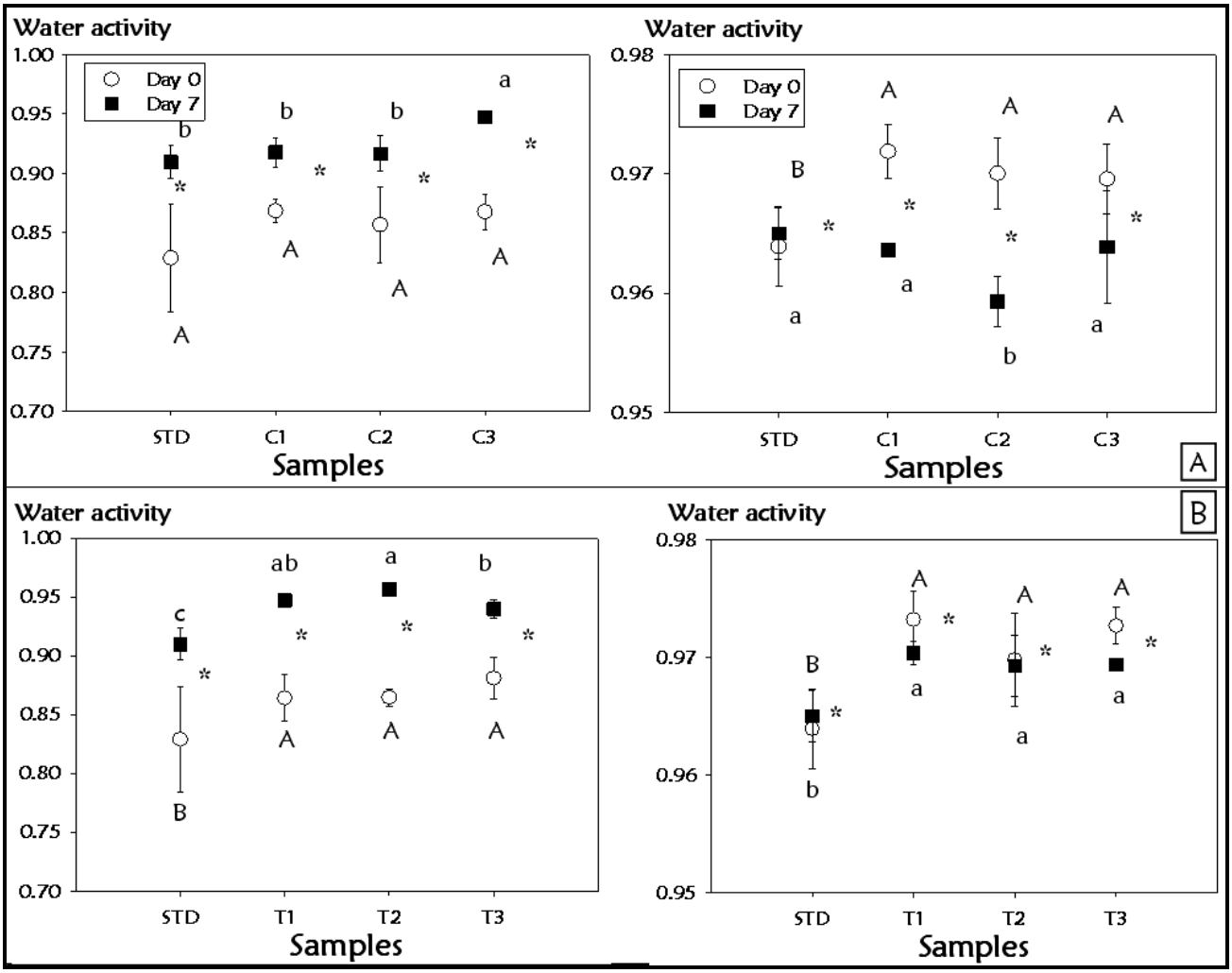


**Figure B 1 - 5:**

**A -** Crust (left) and crumb (right) moisture content of fresh and stored STD, C1, C2, C3

**B -** Crust (left) and crumb (right) moisture content of fresh and stored STD, T1, T2, T3

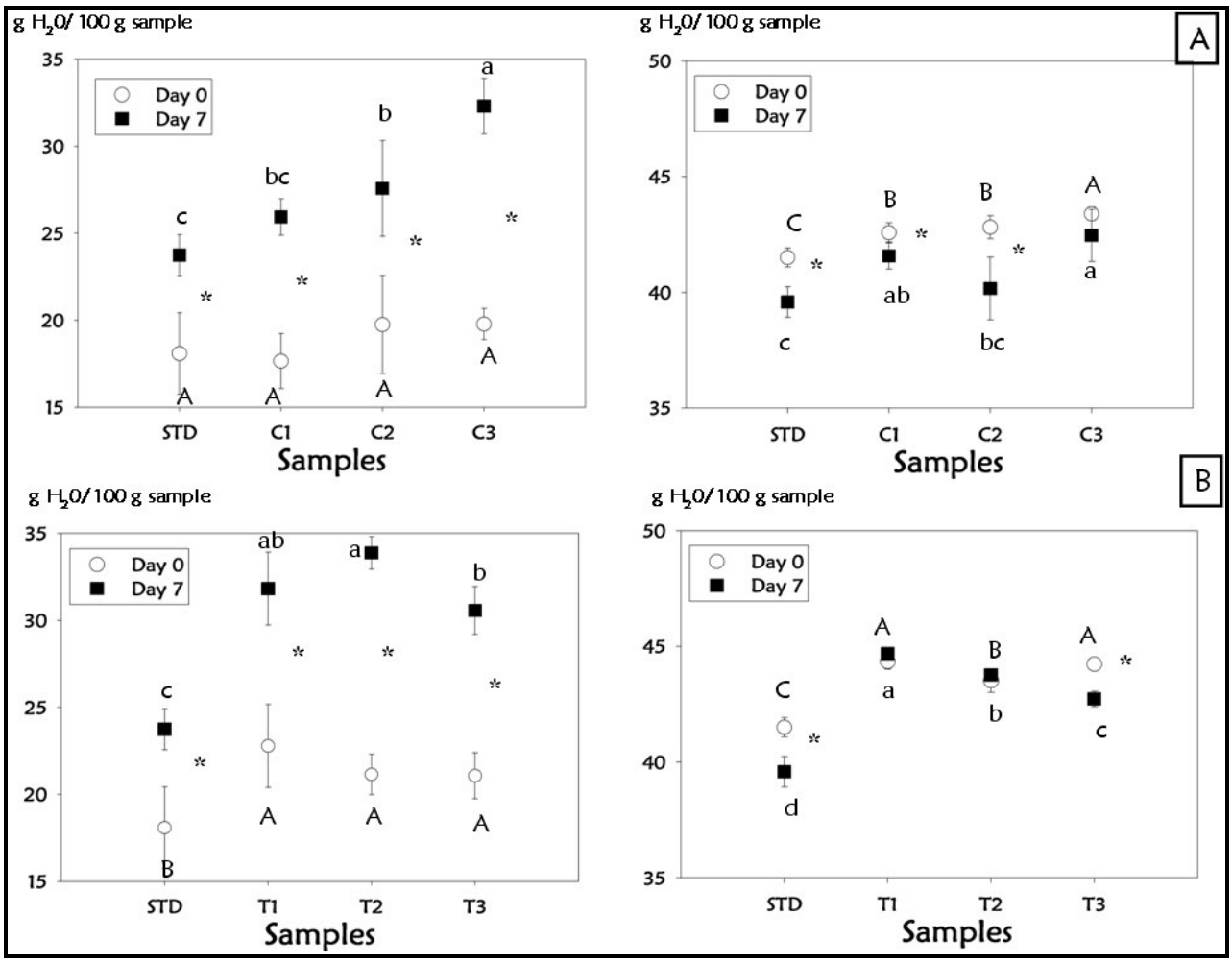


**Figure B 1 - 6:** Crumb frozen water (FW) content of fresh and stored STD, C1, C2, C3 (left) and STD, T1, T2, T3 (right)

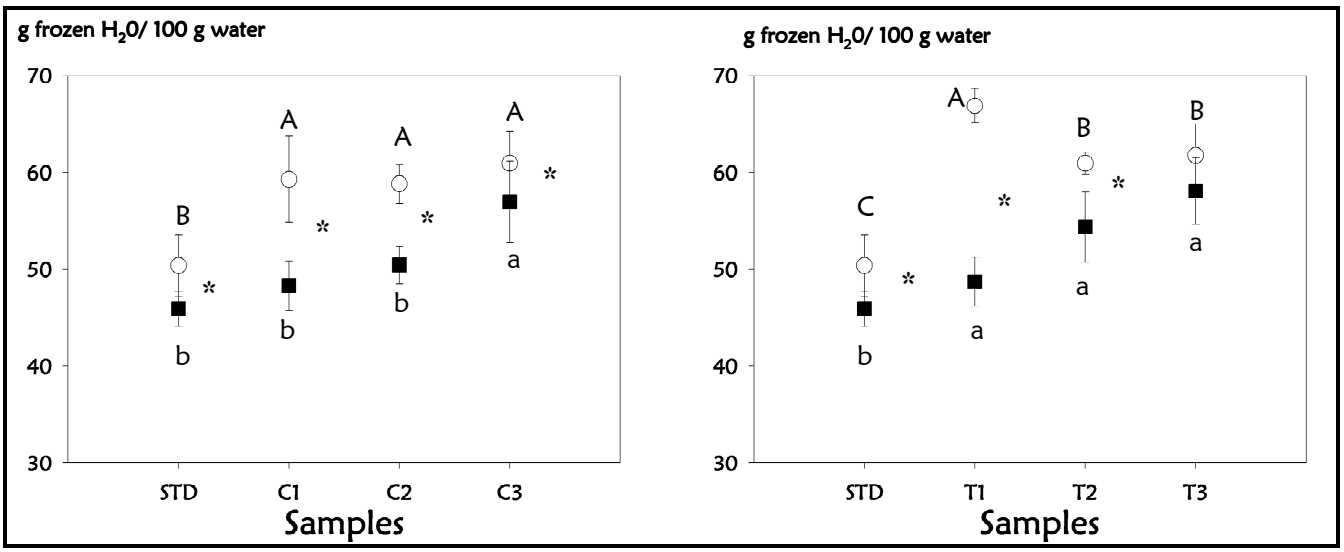



**Figure B 1 - 7:** Melting entalphy of recrystallized amylopectin in stored STD, C1, C2, C3 (left) and STD, T1, T2, T3 (right)

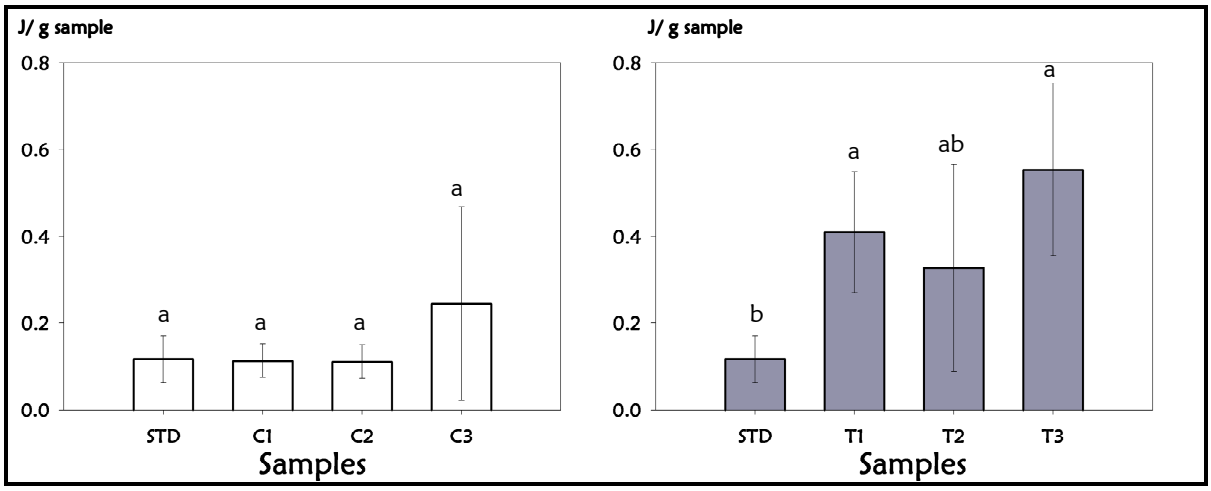


**Figure B 1 - 8:**

**A -** $^{1}$H FID decays for fresh and stored STD, C1, C2, C3

**B -** $^{1}$H FID decays for fresh and stored STD, T1, T2, T3

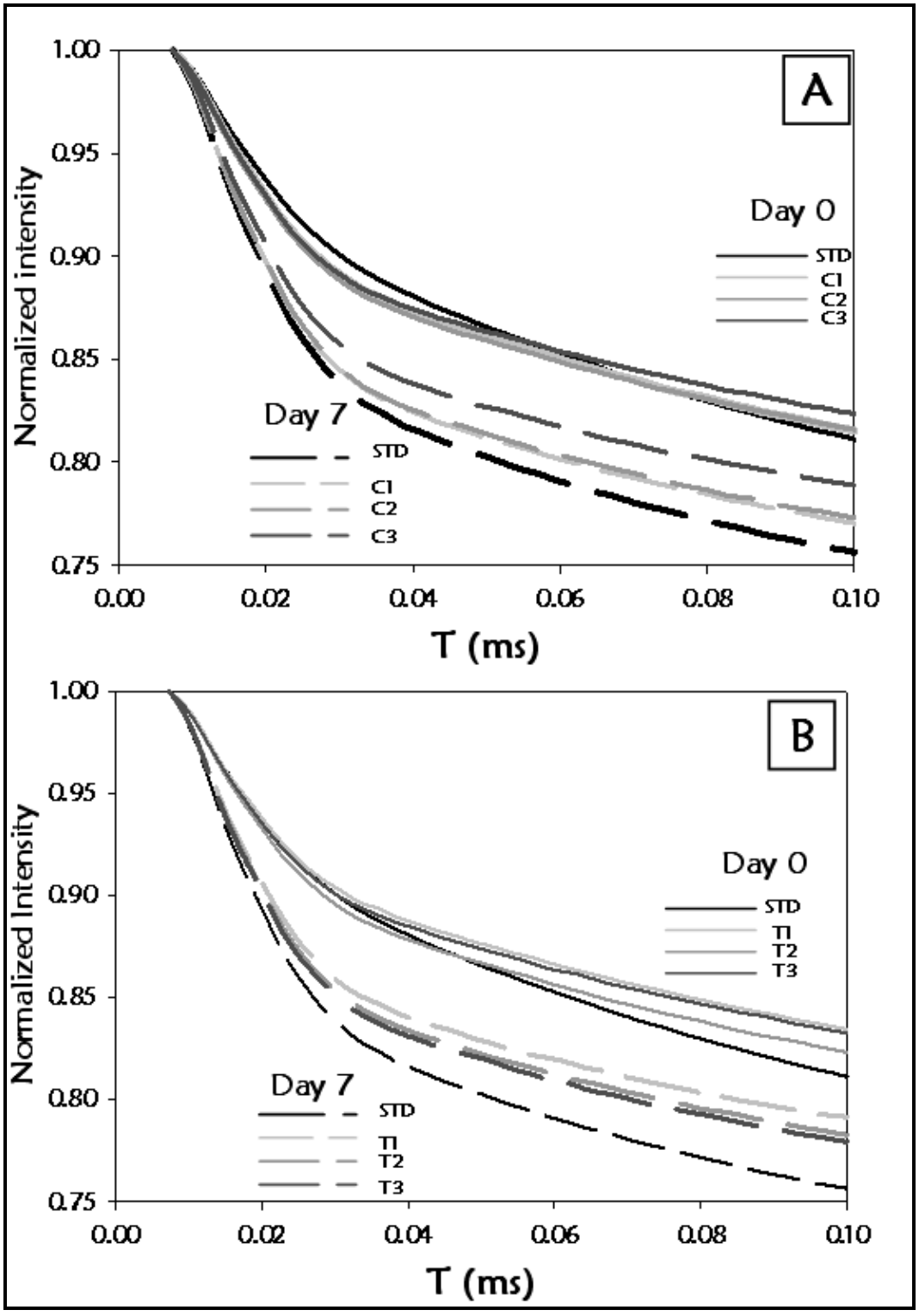


**Figure B 1 - 9:** $^{1}H$ $T_2$ distributions of relaxation times decays for fresh (left) and stored (right) STD, C1, C2 and C3

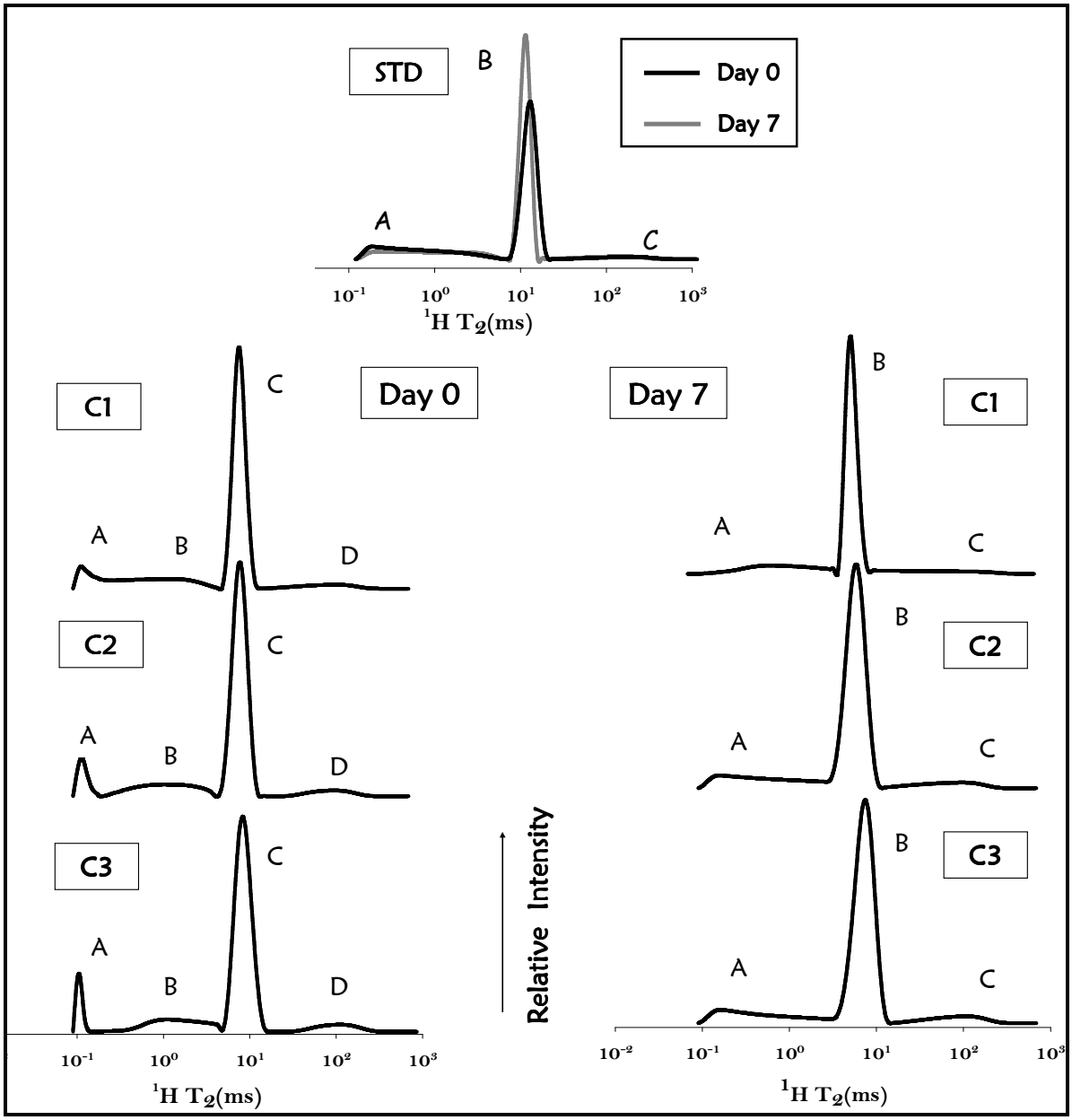
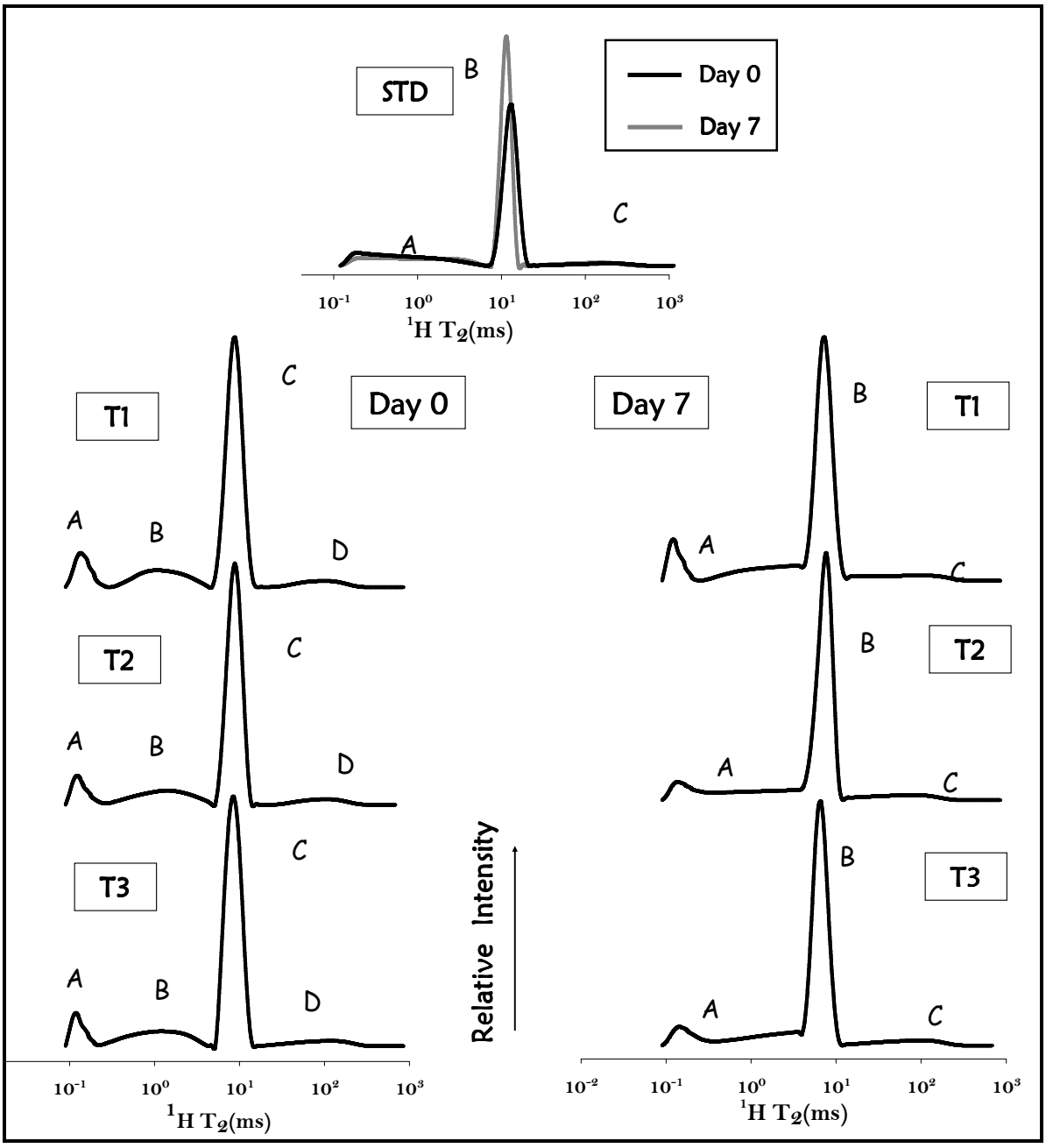


**Figure B 1 - 10:** $^{1}H$ $T_2$ distributions of relaxation times decays for fresh (left) and stored (right) STD, T1, T2 and T3



# Section B 2

## EFFECT OF BRAN AND WHOLEWHEAT FLOUR ON BREAD PROPERTIES AND STALING

Elena Curti, Elena Vittadini, Eleonora Carini

This work was presented at AICAT2008 - XXX National Congress on Calorimetry, Thermal Analysis and Applied Thermodynamics
Pisa, Italy 9-12 December 2008

## 1. Abstract

Bran enriched bread were produced partially substituting wheat flour with bran at three different levels (5, 10 and 20%) and replacing totally wheat flour with whole wheat flour. Bread properties and staling were evaluated at different levels for macroscopic properties (volume, crust colour, crumb structure, texture), macromolecular properties (amylopectin recrystallization) and water status (moisture content, frozen water, $^1$H FID, $^1$H $T_2$ and $T_1$ relaxation time and self diffusion coefficient D) during 3 days of storage. Bran added (samples B5, B10 and B20) affected macroscopic properties as observed for the lower volume and the higher presence of small pores in crumb structure as compared to STD. Hardness was generally comparable to STD in both fresh and stored bran added samples. Water status was strongly affected as an higher crumb moisture content and generally higher frozen water content were found in bran added samples. At a molecular level the addition of bran resulted in increased molecular mobility in fresh samples (higher $^1$H self diffusion coefficient; lower % fast relaxing $T_2$ $^1$Hs - population A and higher % intermediate $T_2$ $^1$Hs - population B) and in a more heterogeneous molecular mobility in stored samples as compared to STD. Whole wheat sample (INT) was harder than all other samples. Lower crumb moisture content (comparable to STD) and lower frozen water content were observed and also an overall reduced molecular mobility in protons population A and B was observed but also an higher abundance (and comparable to B10 and B20) of slowest-relaxing protons. These results indicated a very different effect of the "naturally present bran" (INT) as compared to bran addition (B5, B10 and B20). The obtained results might suggest that a possible weaker water – solids interaction was established in the bran enriched breads while on the contrary a stronger water-solid interaction might have been induced by the natural present bran although not as strong as in the STD sample.

## 2. Introduction

Bran/fibres characteristics and fibre enriched breads have already been discussed in **Section B1, 2. Introduction**.

This work aims to compare the effect of bran on bread properties and staling replacing wheat flour partially with bran fractions and totally with whole wheat flour.

## 3. Materials and methods

### 3.1 Bread formulation, processing and storage

Bread loaves were produced using wheat flour and water (ratio 100:58) sugar 6, yeast 3, salt 2, seeds oil 3, for the control bread (STD) and the fibre enriched breads were produced replacing wheat flour with durum wheat bran at three different levels (5%-B5, 10%-B10 and 20%-B20 on wheat flour basis and substituting the wheat flour at 100% to obtain a whole wheat flour sample (INT). The formulations can be found in Table B 2 - 1.

Bread was produced with a home bread-maker (Silver bread maker XL, Type 1941, Princess®, NL) using the "wholemeal" program (3h 30 min). Bread loaves were allowed to cool to room temperature for two hours prior to be placed in sealed polyethylene bags. Few drops of denaturised alcohol were sprinkled in the bags, samples were stored at 25°C for 3 days, and analysed at 0 and 3 days after production. Two bread loaves were analyzed at each storage time.

### 3.2 Bread Characterization

Bread loaves were characterized for macroscopic (volume, hardness, cohesivity, crust colour), macromolecular (frozen water content, recrystallization of amyolpectin) and water properties (moisture content and molecular mobility) as reported in the section Methods of analysis. Crumb structure was also studied as described in the following paragraph.

#### 3.2.1 Crumb structure

Crumb grain was characterized by enumerating the pores present in five preselected dimensional classes based on their area (class 1  0.001 - 0.005 $mm^2$; class 2 0.005 - 0.01 $mm^2$; class 3 0.01 - 0.1 $mm^2$; class 4 0.1 - 1 $mm^2$; class 5 1 - 3 $mm^2$) and the number of pores and the area occupied by each class (expressed as percentage of the total number of pores) was evaluated.

#### 3.2.2 Statistical analysis

Analysis of variance (ANOVA, post hoc tests: HSD of Tukey and LSD) was used to identify differences among all samples of considered properties at the same storage time (SPSS v.15,

SPSS Inc. IL, USA). Capital letters and small letters indicate significant differences among samples at the same storage time (fresh and stored sample respectively).

## 4. Results and discussion

### 4.1 Macroscopic bread properties

#### 4.1.1 Loaf volume

The volumes of all the considered samples are shown in Figure B 2 - 1.

A relevant decrease of volume was observed in the fibre enriched samples, as the bran content increased. The complete substitution with whole wheat flour (INT) resulted in a comparable volume to the sample richer in bran B20. These results are in agreement with previous studies (Pomeranz et al.,1977; Lai et al., 1989) that reported of a significant reduction of volume when bran fractions were added to white bread, especially when in high amounts (10-14%).

#### 4.1.2 Crumb structure

The scanned images of central slices of all samples and the areas occupied by each class (expressed as percentage of the total number of pores for each class) are reported in Figure B 2 - 2. B5, B10 and B20 showed an higher abundance of small pores belonging to class 1 (0,001 – 0,005 $mm^2$) than INT and STD respectively. A significantly lower abundance of pores belonging to class 2 (0,005 – 0,01 $mm^2$) was found in INT and STD. The fibre enriched samples (B5, B10 and B20) showed a significant higher abundance of pores belonging to class 3 (0.01 - 0.1 $mm^2$). The abundance of larger pores [class 4 (0.1 - 1 $mm^2$) and class 5 ( 1-3 $mm^2$)] was affected by bran addition and whole wheat flour but no correlation was found. INT was found to be generally more similar to STD for the crumb structure, indicating that the total substitution of flour with whole wheat flour affected to a lesser extent crumb porosity than the partial flour substitution.

#### 4.1.3 Crust colour

The L*, a*, b* and $\Delta E$ values of STD, B5, B10, B20 and INT are shown in Table B 2 - 2.

Addition of bran fractions to the bread formulation significantly altered crust colour, as indicated by $\Delta E > 3.5$, as previously reported by Pomeranz et al. (1977). B5 and B10 were distinguishable from STD while B20 and INT were strongly distinguishable from STD.

B5, B10 and B20 resulted significantly different from STD in terms of lower brightness (L*) and generally higher redness (a*). B20 and INT showed a significantly lower yellowness (b*).

### 4.1.4 Crumb texture

Hardness values of all considered samples (fresh and stored) are shown in Figure B 2 - 3.

Hardness of fresh bran enriched samples was comparable to STD and only INT resulted significantly harder. Hardness significantly increased in all samples during storage. B5 and INT were significantly harder at day 3 than STD, B10 and B20, comparable among each other. These results are in contrast with those obtained for the bran enriched breads studied in Section B 1: in the previously considered samples ("C" and "T" samples) a higher hardness in fresh samples as compared to STD was observed (also at 7 days or storage) possibly due to the different composition of these bran fractions.

### 4.1.5 Moisture content

Moisture content of crust and crumb of all samples are shown in Figure B 2 - 4. Fresh samples showed a lower crust moisture content and an higher crumb moisture content, as expected. At day 0 crust moisture content was significantly higher in B20 than in STD, B5 and B10 (comparable among each other) and INT. Crumb moisture content resulted significantly higher in B20, followed by B5 and B10 (comparable between each other) as compared to STD and INT (comparable between each other). These results confirmed the previous findings reported in Section B 1, where the moisture content was found to be generally higher in the bran enriched breads.

During storage crust moisture content significantly increased, as a consequence of the macroscopic migration of water occurring from the wetter crumb to the drier crust and was comparable among all stored samples, except for significantly lower values observed in B20. No significant decrease in crumb moisture content was observed, possibly due to the short storage period considered.

## 4.2 Macromolecular bread properties

### 4.2.1 Thermal analysis

The characteristic DSC thermograms exhibited two endothermic transitions (with the exception of STD and INT) as the samples were heated from −80 to 130°C. A first major endothermic event was observed around 0°C and a second minor endothermic event occurred at higher temperatures (50–80°C).

The major DSC endothermic peak was attributed mainly to ice melting (Vodovotz et al.,1996; Li, Dickinson and Chinachoti, 1998; Baik and Chinachoti, 2001) in all samples. FW values of all samples are shown in Figure B 2 - 5. At day 0 the frozen water content (FW) was comparable among the bran enriched samples and significantly lower in STD and INT.

Frozen water content generally decreased in all samples during storage, due to the migration of a fraction of water from gelatinized starch towards the more rigid amorphous and crystalline domains that so became unfreezable Baik and Chinachoti, 2000; Hallberg and Chinachoti, 2002; Ribotta and Bail, 2007; Kerch et al. 2008), and it was comparable among all stored samples.

The enthalpy values of the endothermic peak in the temperature range 60-80°C for fresh and stored samples are shown in Figure B 2 - 5. The peak area, indicating the melting of crystalline amylopectin as previously reported (Russell, 1983), was also observed in fresh samples B5, B10 and B20. The enthalpy increased in all samples and it was significantly lower in INT and B10. The presence of an endothermic peak in fresh samples with bran may indicate that the starch did not undergo a complete gelatinization during cooking in these sample and/or a very fast amylopectin recrystallization may have occurred.

## 4.3 Molecular properties

Molecular characterization was carried out with multiple $^1H$ NMR experiments to cover a large range of molecular relaxation events. $^1H$ rotational mobility was studied, at 20 MHz, for the fastest-relaxing component, with a FID experiment while the slower relaxing proton fractions were characterized in terms of $T_2$ and $T_1$ relaxation times distributions. Translational $^1H$ molecular mobility was quantified in terms of the $^1H$ self diffusion coefficient. $^1H$ FID decays of fresh and stored samples are shown in Figure B 2 - 6: the first, fast relaxing portion of the FID decay (<0,08 ms) is indicative of the presence of a very rigid $^1H$ population. $^1H$ FID decays of INT and the bran enriched samples were slower than STD $^1H$ FID at day 0, indicating an higher molecular mobility. As expected, the $^1H$ FID rigid component became progressively more relevant during storage in all samples, due to a reduced mobility of the bread matrix due to both recrystallizing amylopectin and loss of water from the crumb. It was observed that STD underwent a more marked loss in mobility than all the other samples.

The $^1H$ $T_2$ distributions obtained using an UPEN software were analyzed for $T_2 \geq 0.089$ ms (2 interpulse spacing + instrument dead time) to avoid extrapolation of $T_2$ values at times shorter than the first point measured with the CPMG experiment. $^1H$ $T_2$ quasi-continuous distributions of all samples (fresh and stored) are shown in Figure B 2 - 7. Three $^1H$ $T_2$ populations were observed in all fresh samples (except for B20) and they were named starting from the shorter to the longest relaxation time A, B and C, respectively. A represented a population of protons characterized by relaxation times at ~0.2 ms ($T_{2A,}$ peak

time); the B protons relaxed at ~ 10 ms range ($T_{2B}$, peak time); C protons were characterized by relaxation times around 100 ms ($T_{2C}$, peak time). Two $^1H$ $T_2$ populations were observed in B20: the first one resulted from the overlapping of A and B populations observed in the other samples and was characterized by relaxation times around 10 ms ($T_{2B}$) and the second one was comparable to population C. In fresh samples population A was significantly higher in STD (27.6 ± 0.5%) than in INT and B5 (26.1 ± 0.5% and 25.7 ± 0.6% respectively) and B10 (24.1 ± 0.6%); population B was significantly lower in STD and INT (68.4±0.7% and 68.6±1.3% respectively) than in B5 (69.6 ± 0.5%) and B10 (70.8± 0.6%); population B was comparable in STD and B5 (3.9±0.5% and 4.6±0.5% respectively) and higher than in other samples (>5%). $^1H$ $T_2$ relaxation times $T_{2A}$ and $T_{2C}$ were generally comparable among all samples (12 ms) while $T_{2B}$ was significantly lower in the bran enriched bread (~ 10 ms) and in INT (~ 9 ms). $^1H$ $T_2$ relaxation times distributions slightly shifted towards lower relaxation times in all samples.

At 3 days of storage, STD and B5 showed a significant decrease of population A (23.1 ± 1.4% and 22.5±1.2% respectively), a significant increase of population B (72.0 ± 1.2% and 71.8±0.8% respectively) while population C remained constant. Only two populations were found in B10, B20 and INT: a broader proton population (AB), characterized by two peak times ($T_{2AB1}$ ~0.15 ms and $T_{2AB2}$ ~9 ms), encompassing ~94% of the total protons and a smaller population (C, $T_{2C}$ ~95-100 ms). It is likely that the $^1H$ population AB resulted from the overlapping of the A and B proton populations that at longer storage times were no longer resolved (clearly separated) as the protons underwent exchange within the NMR experimental time-frame.

The $^1H$ $T_2$ distribution of relaxation times of these samples resulted different to those observed in the bran added samples studied in Section B 1. The fourth $T_2$ protons population found in "C" and "T" samples in Section B 1 was not observed here, indicating that the bran fractions in B5, B10 and B20 may have differently altered the water distribution between the gluten matrix and the starch phase. The different composition of these bran fractions resulted in $^1H$ $T_2$ distributions of relaxation times more similar to STD in fresh samples, suggesting that their composition may have affected differently water-solids interactions at a molecular level.

Proton $T_1$ distributions of all samples were unimodal and representative of a unique proton population. The relaxation times, representative of the major peak of $^1H$ $T_1$ distributions, for fresh and stored samples are shown in Table B 2 - 3. In fresh samples $^1H$ $T_1$ relaxation times were significantly higher in B20 and INT. During storage $^1H$ $T_1$ relaxation times increased in

all samples except for INT. This result is in disagreement with previous studies (Chen et al. 1997) that observed a decreased in T1 values attributable to an overall decrease of molecular mobility.

The self diffusion coefficients of all samples are shown in Table B 2 - 4. In stored samples, STD showed significantly lower $^1H$ $T_1$ relaxation times than the bran enriched samples.

## 5. Conclusions

Bran enriched bread were produced partially substituting wheat flour with bran at three different levels (5, 10 and 20%) and replacing totally wheat flour with whole wheat flour. Bran enriched samples (B5, B10 and B20) showed lower volumes and a higher presence of small pores in crumb structure as compared to STD. Bran enriched samples were comparable to STD for hardness both fresh and stored. Higher crumb moisture extractable at 105°C and generally higher frozen water content were found in B5, B10 and B20 than in STD. At a molecular level the addition of bran resulted in an increased molecular mobility in fresh samples (higher $^1H$ self diffusion coefficient; lower % fast relaxing $T_2$ $^1Hs$ - population A and higher % intermediate $T_2$ $^1Hs$ - population B). Bran-enriched samples showed $^1H$ $T_2$ distributions of relaxation times where protons populations were not longer resolved as in fresh samples, indicating an heterogeneous molecular mobility. These results might suggest a possible weaker water – solids interaction in bran enriched breads, due to the competition of bran for water. The bread containing naturally present bran (whole wheat flour – INT) resulted different from the bran-enriched breads. INT showed lower volume and significantly higher hardness than all other samples. Lower crumb moisture extractable at 105°C (comparable to STD) and lower frozen water content in respect to B10 and B20 were observed, suggesting a possible stronger water-solid interaction but not as stronger as in STD. In fact, at a molecular level INT showed an overall reduced molecular mobility in protons population A and B but also higher abundance (and comparable to B10 and B20) of slowest-relaxing protons.

## 6. List of Tables

**Table B 2 - 1:** Bread formulations

| Ingredients (g) | STD | B5 | B10 | B20 | INT |
|---|---|---|---|---|---|
| Wheat flour | 100 | 95 | 90 | 80 | / |
| Whole wheat flour | / | / | / | / | 100 |
| Bran | / | 5 | 10 | 20 | / |
| Sugar | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 |
| Salt | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 |
| Yeast | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 |
| water | 58 | 60.3 | 63 | 69.2 | 57.4 |
| Sunflower seeds oil | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 |
| Wheat flour (0) | 100 | 95 | 90 | 80 | 100 |

**Table B 2 - 2**: Bread crust colour attributes

| | STD | B5 | B10 | B20 | INT |
|---|---|---|---|---|---|
| L* | $62.9 \pm 2.7^{a}$ | $60.3 \pm 1.8^{ab}$ | $58.3 \pm 3.3^{b}$ | $49.5 \pm 3.2^{b}$ | $58.1 \pm 3.3^{c}$ |
| a* | $9.5 \pm 1.2^{bc}$ | $8.2 \pm 2.4^{b}$ | $10.6 \pm 1.9^{ab}$ | $11.8 \pm 2.1^{a}$ | $7.8 \pm 0.6^{dc}$ |
| b* | $31.5 \pm 1.4^{a}$ | $27.5 \pm 2.5^{bc}$ | $29.2 \pm 2.5^{b}$ | $26.3 \pm 2.4^{c}$ | $17.4 \pm 1.5^{d}$ |
| ΔE* | / | **4.9** | **5.2** | **14.5** | **15.1** |

**Table B 2 - 3**: $^{1}H$ $T_1$ relaxation times of fresh and stored samples

| $^{1}H$ $T_1$ (ms) | Day 0 | Day 3 |
|---|---|---|
| STD | 99.57± 0.84 | 114.91± 13.01 |
| B5 | 102.95± 1.77 | 107.33± 2.15 |
| B10 | 99.09 ±0.73 | 115.92± 10.45 |
| B20 | 104.09± 4.52 | 113.86± 3.09 |
| INT | 121.98 ±8.63 | 99.93± 1.73 |

**Table B 2 - 4**: $^{1}H$ Self diffusion coefficient (D) of fresh and stored samples

| D ($*10^{-9}$ $m^2*s^{-1}$) | Day 0 | Day 3 |
|---|---|---|
| STD | $0.460\pm0.011^{b}$ | $0.487\pm0.025^{b}$ |
| B5 | $0.471\pm0.027^{ab}$ | $0.469\pm0.019^{b}$ |
| B10 | $0.469\pm0.012^{ab}$ | $0.488\pm0.016^{b}$ |
| B20 | $0.482\pm0.024^{a}$ | $0.515\pm0.019^{b}$ |
| INT | $0.417\pm0.018^{c}$ | $0.426\pm0.015^{b}$ |

## 7. List of figures

**Figure B 2 - 1:** Volumes of samples

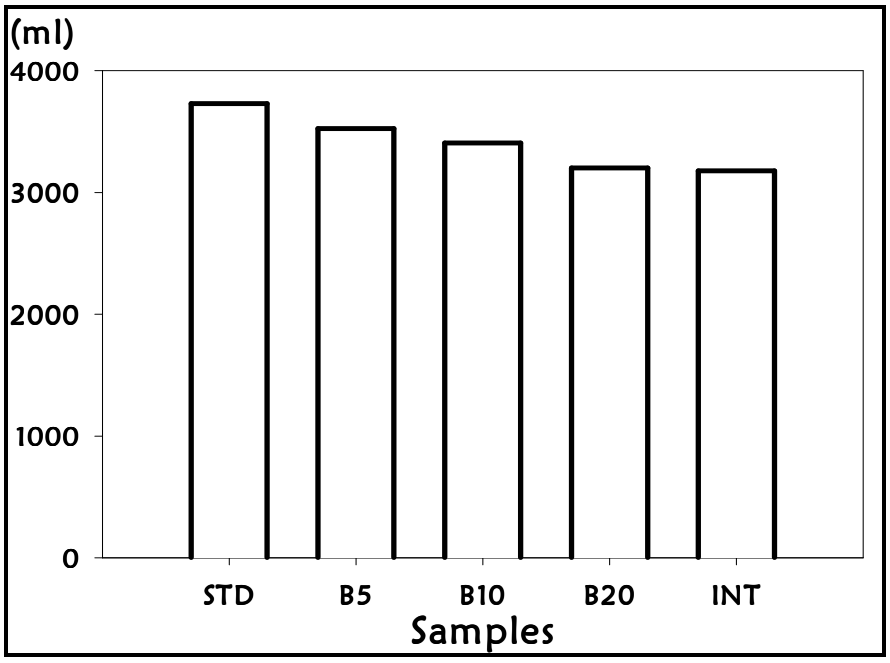


**Figure B 2 - 2:** Abundance (%) of pores in 5 classes of area

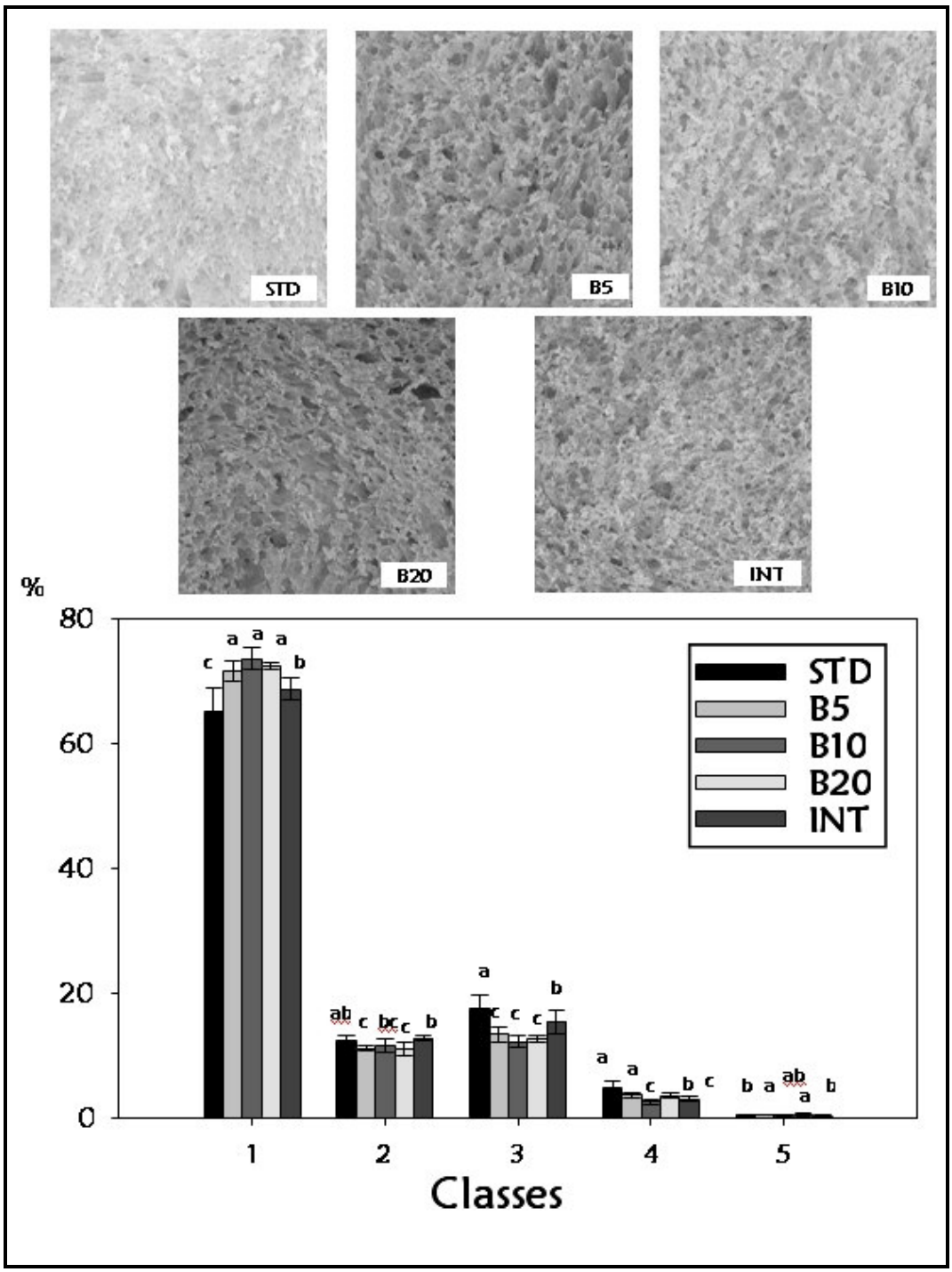


**Figure B 2 - 3:** Crumb hardness (left) and cohesivity (right) of fresh and stored samples

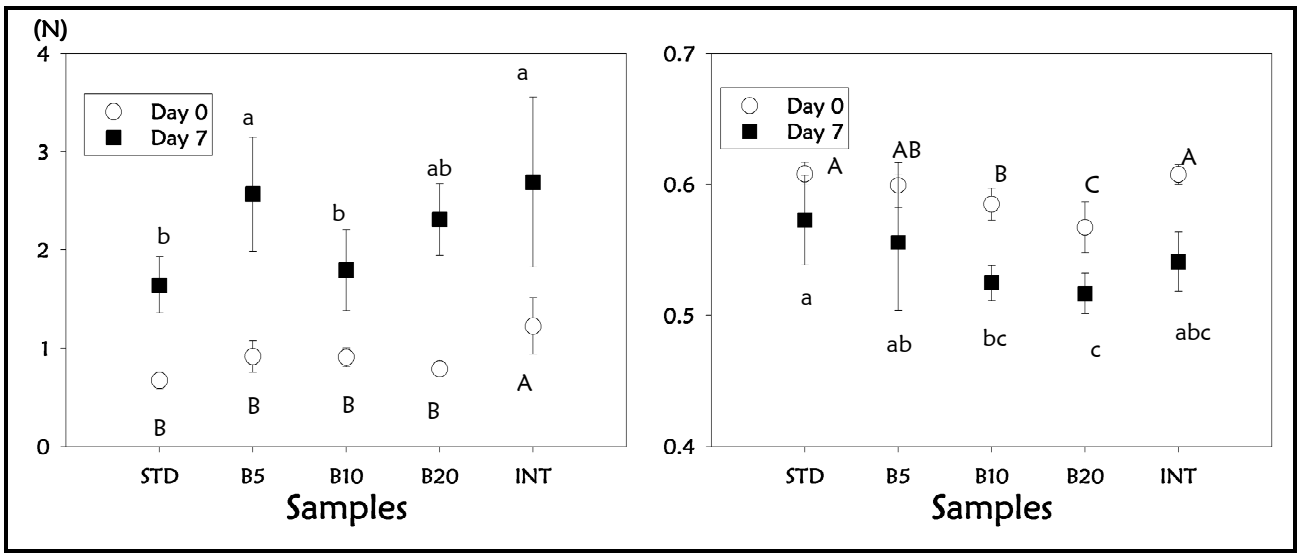



**Figure B 2 - 4:** Crust (left) and crumb (right) moisture content of fresh and stored samples

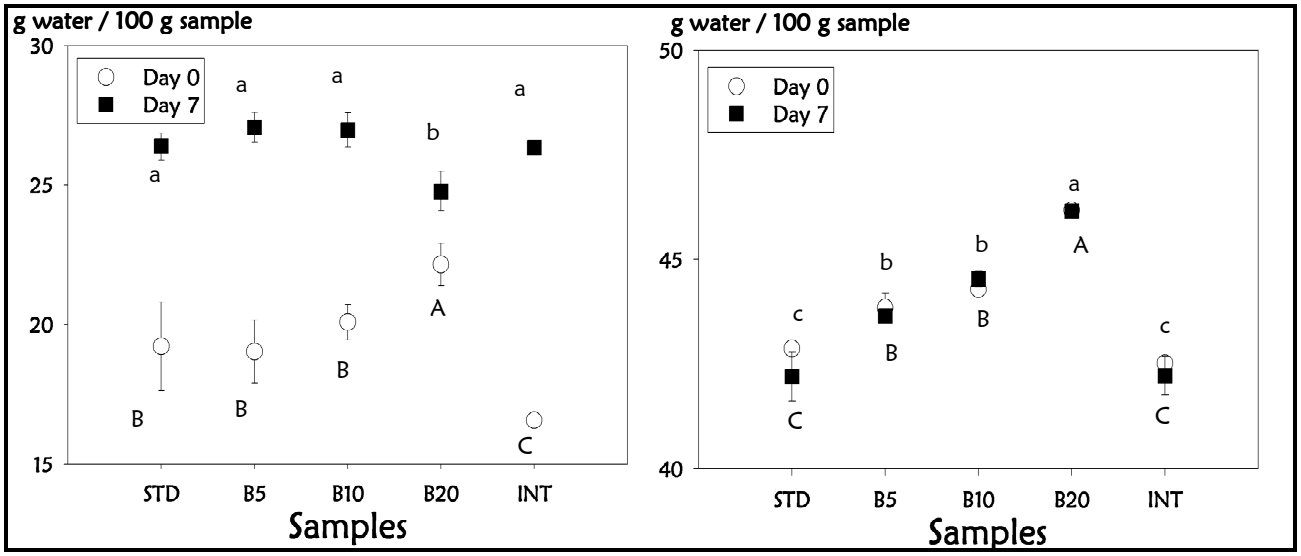


**Figure B 2 - 5:** Crumb frozen water content (left) and melting entalphy of recrystallized amylopectin (right) in fresh and stored samples

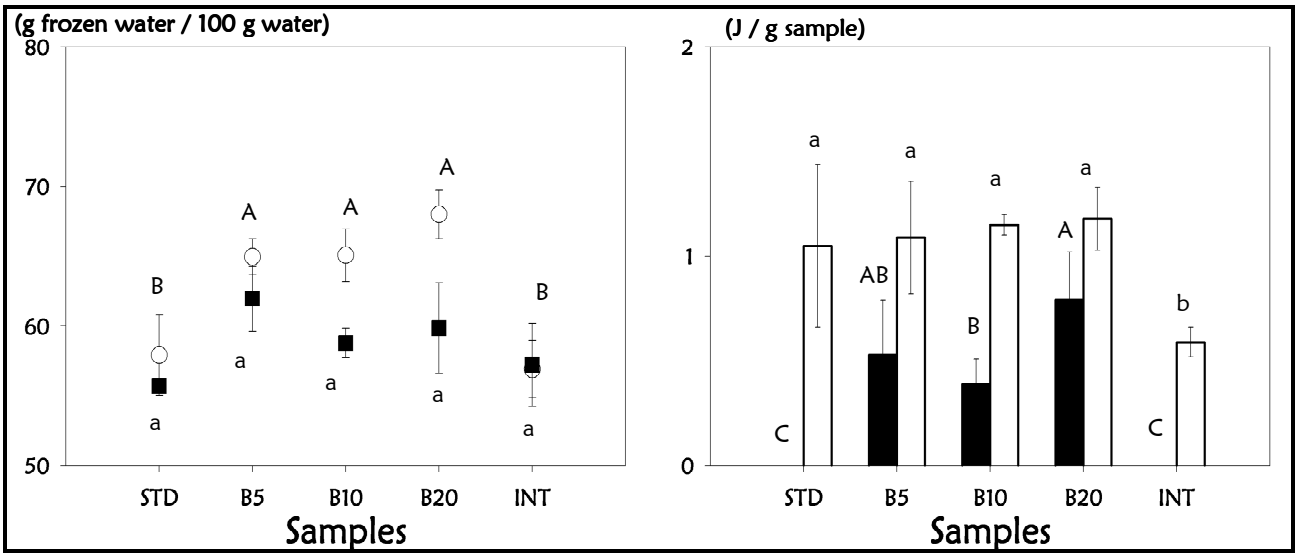


**Figure B 2 - 6:** $^{1}H$ FID decays for fresh and stored samples

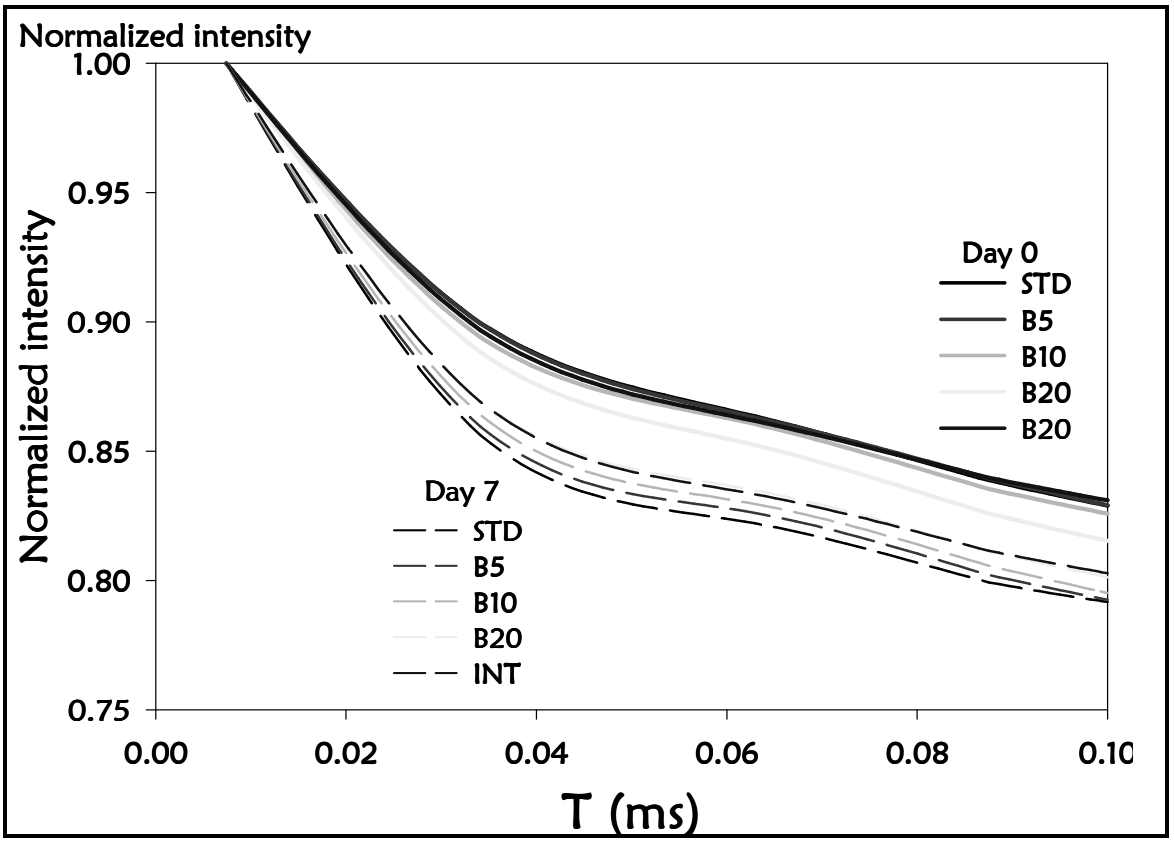
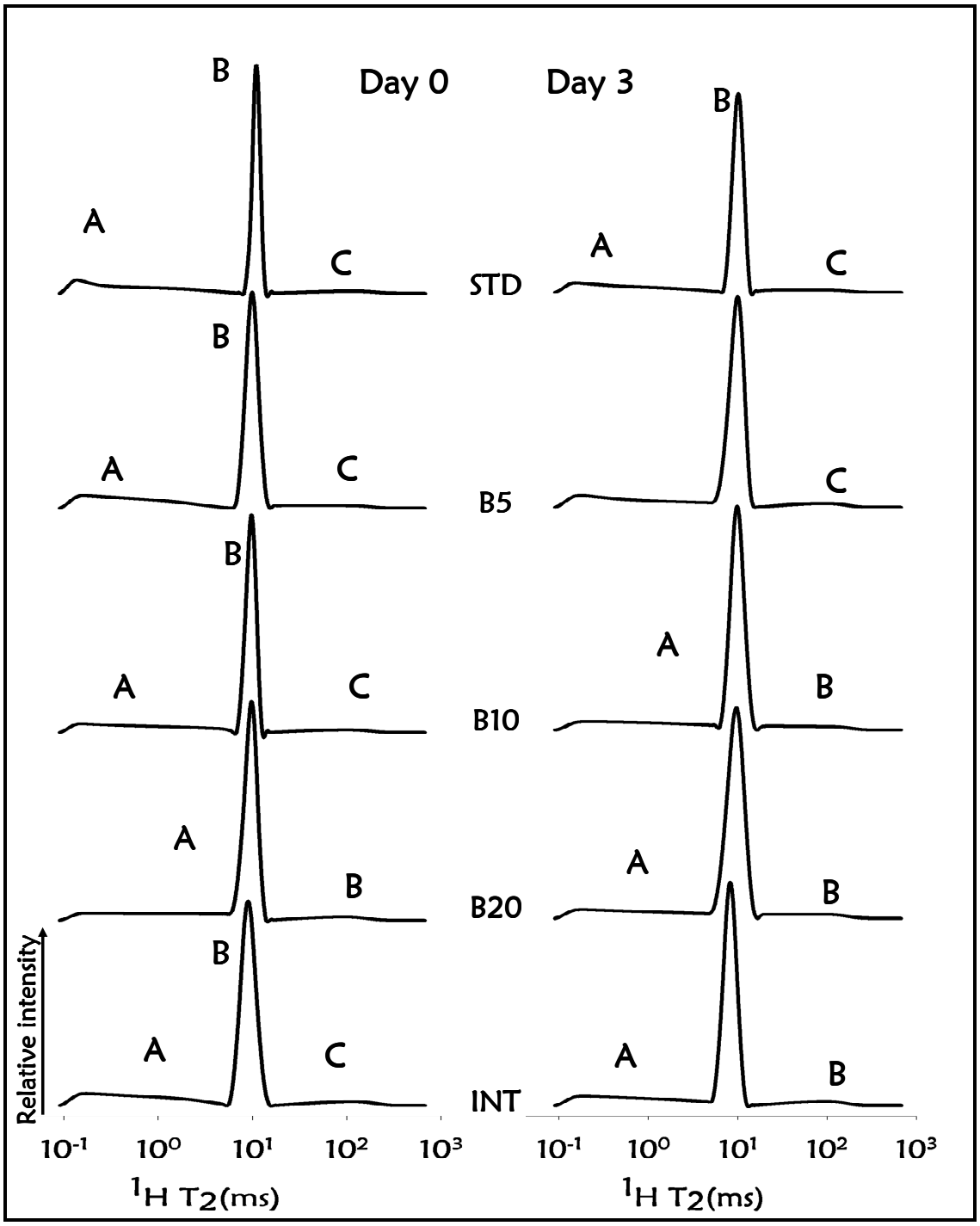


**Figure B 2 - 7:** $^{1}H$ $T_2$ distributions of relaxation times decays for fresh (left) and stored (right) samples



# Section C: NMR TECHNIQUES

# MULTI-LEVEL ANALYSIS OF BREAD STALING

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## 1. Abstract

Bread staling is a complex phenomenon that originates from multiple physicochemical events (including amylopectin retrogradation, water loss and water molecular redistribution) and it is not yet completely elucidated. White bread loaves were characterized at different levels for physicochemical properties during 14 days of storage (hardness, amylopectin recrystallization) and water status (moisture content, frozen water, $^1$H FID, $^1$H $T_2$ and $T_1$ relaxation time and $^1$H $T_1$ relaxation times at variable frequency with a Field Cycling Spectrometer). Macroscopic changes were observed during the period of storage (migration of water molecules from the crumb to the crust, hardening of the crumb). Macromolecular changes were detected as evidenced by the decrease frozen water content and the increase in the amylopectin recrystallization. At a molecular level, a faster decay of $^1$H FID curves and a shifting of $^1$H $T_2$ distributions of relaxation times towards shorter times indicated a $^1$H mobility reduction of the bread matrix. Multiple $^1$H $T_2$ populations of protons were observed and tentatively associated to water-gluten and water-starch phases, that are known to undergo molecular changes during storage that play an important role in bread staling. $^1$H $T_1$ was investigated at different frequencies: in particular a decreased in $^1$H $T_1$ mobility was evidently observed at frequencies lower than 0.2 MHz, suggesting that $^1$H NMR techniques operating in the frequency range 0.01–0.2 MHz allow to highlight mobility changes that are not well detectable at 20 MHz.

## 2. Introduction

Staling is defined as the "decreasing consumer acceptance of bakery products caused by changes in crumb other than those resulting from the action of spoilage organisms" (Bechtel et al., 1953). Although bread staling has been studied for more than 150 years, the molecular basis of this phenomenon are, to date, not completely understood (Gray and

Bemiller, 2003). Bread staling is a time-dependent process that originates from multiple concurrent physico-chemical events resulting in crumb hardening, crust softening and loss of characteristic fresh flavour of the product. Starch is known to undergo retrogradation during storage: amlylose retrogradation is very fast and can be considered completed upon cooling of the product (Kim and D'Apollonia, 1977) while amylopectin recrystallization occurs over longer times (days-weeks). Starch, and, in particular, amylopectin retrogradation is only partially responsible of the changes in bread properties during storage (Hallberg and Chinachoti, 2002, Vodovotz, Vittadini and Sachleben, 2002). Water has been reported to play an important role in bread staling, not only because it undergoes a macroscopic migration from crumb and crust (Lin and Lineback, 1990, Schiraldi and Fessas, 2001; Baik and Chinachoti, 2001) but also because it undergoes significant changes (mobility, interaction with other molecules) at a molecular level that can affect macromolecular dynamics. Water molecules have been reported to partially become incorporated in retrograded amylopectin crystals (Imberty and Perez 1988), to loose phase separating capability (decreased "DSC freezable water" content (Slade and Levine 1991; Vodovotz, Hallberg and Chinachoti, 1996; Vittadini and Vodovotz, 2003), to migrate from gluten to starch causing a plasticity/elasticity loss of the continuous phase of the bread crumb (Leung, 1981, Slade and Levine, 1991; Callejo, Gill ,Rodriguez and Ruiz, 1999), to reduce molecular mobility (Chen, Long, Ruan and Labuza, 1997; Vodovotz et al., 2002; Sereno, Hill, Mitchell, Scharf and Farhatt, 2007).

The bread staling process is, therefore, a very complex phenomenon that encompasses multiple events that may take place simultaneously and/or sequentially and that may involve phenomena occurring at different time-space scales in the bread matrix. Hence the study of bread staling should be carried out with a multi-analytical approach to characterize physico-chemical changes (ranging from molecular to macroscopic) occurring in the bread material. Particular attention should be given to the characterization of the status and the dynamics of water since water is directly or indirectly involved in many phenomena occurring during bread staling as reported above. Water activity, moisture content and frozen water content measurements gave information about averaged and long range water properties (Vittadini, Dickinson and Chinachoti, 2001 and 2002; Vittadini and Chinachoti, 2003; Vittadini, Clubbs, Shellhammer and Vodovotz, 2004) and a different perspective of the molecular properties of water was obtained using NMR to investigate the dynamics of food materials at a molecular level. Although $^{1}H$ NMR spectroscopy is not a specific probe for water (Halle and Wennerstroem, 1981; Schmidt and Lai, 1991; Colquhoun and

Goodfellow, 1994; Ruan and Chen, 2001), the mobility of food components is strongly dependent upon their interaction with water and the observed $^1$H NMR signal encompasses also the contribution of other proton species closely interacting with water and of the molecular dynamics existing among the protons in different domains.

Low resolution $^1$H NMR spectroscopy has been previously applied to bread staling studies using multiple experiments to observe different windows of relation times. The fastest-relaxing protons can be monitored with $^1$H FID experiments; it was previously reported that the $^1$H FID rigid component (measured at 23MHz) became progressively more relevant during storage both in gelatinized waxy maize starch (Farhat, Ottenhof, Marie and de Bezenac, 2003) and bread (Sereno et al. 2007). This $^1$H FID rigidity increase was attributed to a reduced mobility of the bread matrix due to both recrystallizing amylopectin and loss of water from the crumb. The slower relaxing proton fractions were characterized in terms of $^1$H $T_2$ and $T_1$ relaxation times distributions. In particular, $^1$H $T_2$ relaxation has been reported to well represent mobility changes of baked products over storage by low resolution NMR experiments conducted by Engelsen, Jensen, Pedersen, Norgaard and Munckl (2003), Sereno et al. (2007) at 23MHz and Chen et al. (1997) at 20MHz. These studies reported multiple $^1$H $T_2$ populations in baked products that underwent major changes over storage, resulting in a reduced mobility (shorter $^1$H $T_2$ relaxation time) in stored products. $^1$H $T_1$ relaxation was reported to have a mono-exponential behaviour (at 20MHz) and to slightly decrease in mobility (from ~90 to 80 ms) over storage (Leung, Magnuson and 1993; Chen et al, 1997), providing a less informative insight on the molecular dynamics of bread staling.

A new approach in the analysis of macromolecular dynamics in bread staling may be represented by the fast field cycling techniques. FFC applies a variable magnetic field to the sample allowing for the measurement of longitudinal relaxation times at different frequencies and, consequently, widening the range of $^1$H molecular motions that can be observed. In particular, at low frequencies, it is possible to focus on molecular dynamics characterized by very long correlation times, such as molecular surface dynamics and collective effects (Baroni, Bubici, Ferrante, and Aime, 2009). Nuclear Magnetic Resonance Dispersion (NMRD) profiles [$1/(^1\text{H}\ T_1) = {}^1\text{H}\ R_1$ f(frequency)] are particularly valuable to assess the interactions of water molecules with paramagnetic and large-sized macromolecular systems (Baroni et al. 2009). In particular, the relaxation profile is dominated by the magnetic field dependence of rotationally immobilized protons, dynamically coupled to the spin-lattice relaxation of water protons. The FFC technique has

been previously applied to food matrices: Godfroy, Korb, Creamer, Watkinson and Callaghant (2003) studied two different types of cheese (mozzarella and gouda) investigating the interaction of water with milk proteins upon aging and they reported that the relaxation rate was directly related to the degree of proton hydration that increased with ripening and this phenomenon was better resolved at frequencies < 0.5MHz. Laghi, Cremonini, Placucci and Sykora (2005) clearly observed an increase in $^1H$ $R_1$ at 2-3 MHz in egg albumen during storage resulting from water loss or water redistribution among egg components. Baroni , Consonni, Ferrante and Aime (2009) used FFC as a tool to detect counterfaction of traditional balsamic vinegar of Modena. The presence of paramagnetic ions in the genuine balsamic vinegars samples dominated the relaxation and allowed for differentiation between genuine and counterfeit samples.

In this work bread staling of white bread was analyzed over a wide range of time-space domains ranging from molecular (low resolution NMR spectroscopy) to macroscopic (crumb hardness) and, in particular, the $^1H$ NMR FFC technique was applied for the first time to bread to follow the changes in $^1H$ $T_1$ relaxation in the 0.01 – 20 MHz frequency range during storage.

## 3. Materials and methods

### 3.1 Bread formulation, processing and storage

Bread was produced using the following formulation expressed on a flour basis: wheat flour (100), water (58) sugar (4) yeast (3), sunflower oil (3), and salt (2). Bread loaves were produced with a home bread-maker (Severin BM3986, Germany) using the "wholemeal" program. Bread loaves were allowed to cool to room temperature for two hours prior to be placed in sealed polyethylene bags. Few drops of denaturised alcohol were sprinkled in the bags, samples were stored at 25°C for 14 days, and analysed at 0, 1, 3, 5, 7 and 14 days after production. Three bread loaves were analyzed at each storage time.

### 3.2 Bread characterization

Bread loaves were characterized for macroscopic (volume, hardness), macromolecular (frozen water content, recrystallization of amyolpectin) and water properties (water activity, moisture content and molecular mobility) as reported in the section "Methods of analysis". Crumb structure and molecular mobility by Fast Field Cycling $^1H$ NMR were also studied as described in the following paragraphs.

### 3.2.1 Crumb structure

Crumb grain was characterized by enumerating the pores present in five preselected dimensional classes based on their area: class 1 0.009–0.02 $mm^2$; class 2 0.02–0.05 $mm^2$; class 3 0.05- 0.1 $mm^2$; class 4 0.1-1 $mm^2$; class 5 1-5 mm2. The number of pores and the area occupied by each class (expressed as percentage of the total number of pores) was evaluated.

### 3.2.2 Molecular properties - Fast Field Cycling ($^1$H NMR)

$^1$H NMRD profiles were recorded at 25°C on a Stelar Spinmaster- FFC field cycling relaxometer (Stelar S.r.l., Mede (PV), Italy) by measuring water proton longitudinal relaxation rates at magnetic field strengths in the range from $2.4 \times 10^{-4}$ to 0.25 T (corresponding to 0.01-10 MHz proton Larmor frequencies). The relaxometer was able to switch the magnetic field strength in the millisecond time scale by working under complete computer control with an absolute uncertainty in $1/T_1$ of ±1%. The temperature was controlled by a Stelar VTC-91 airflow heater (Stelar S.r.l., Mede (PV), Italy), equipped with a copper-constantan thermocouple; the actual temperature in the probe head was measured with a Fluke 52 k/j digital thermometer (Fluke AG, Zurich, Switzerland), with an uncertainty of ±0.3°C. 4 scans were used for each acquisition. Relaxation curves were acquired with 64 log-spaced taus ranging from 0.001 to 0.3 s and 512 data points and elaborated as quasi-continuous distributions of relaxation times using a UPEN software as previously reported.

### 3.2.3. Statistical analysis

Significant changes of considered properties were evaluated during storage bread loaves with analysis of variance ANOVA and Tukey - HSD at a 95% confidence level (SPSS, v. 15, IL, USA).

## 4. Results and discussion

Bread loaves were produced and characterized during storage with multiple analytical techniques in an attempt to describe the changes occurring in the material over a wide range of time-space domains ranging from molecular to macroscopic.

Macroscopic characterization of bread loaves indicated that crust of fresh loaves had a moisture content of ~17 % (g $H_2O$ / 100 g sample) and crumb moisture content was ~43 % (g $H_2O$ / 100 g sample; Figure C - 1A). Moisture content of crumb decreased during storage and moisture content of crust increased due to the macroscopic moisture migration from the wetter bread crumb to the drier bread crust (Kulp and Ponte, 1981,; Baik and Chinachoti, 2001). Bread crumb moisture content significantly decreased to ~36 % (g $H_2O$ / 100 g sample) while crust moisture content significantly increased to ~27 % (g $H_2O$ / 100 g sample) during storage (Figure C - 1A). Moisture content of bread crust changed more markedly and faster (significant change after 1 day of storage) as compared to the smaller and slower moisture content change of the crumb (significant change at 7 days of storage) as expected given the much larger amount of bread crumb as compared to bread crust that is found in bread. It is likely that the significant increase in crust moisture in 1-day stored samples could be attributed to water molecules migrating from the nearest portion of crumb while migration of water molecules belonging to the centre of the loaf was better shown at longer storage (~ 36 %, moisture content at 14 days, Figure C - 1A).

Crumb hardness was found to steadily increase during storage from ~0.76 N (day 0) to ~3.4 N (day 14), as expected (Figure C - 1B).

Macromolecular characterization was carried out by Differential Scanning Calorimetry. DSC thermograms (not reported) showed a major DSC endothermic peak around 0°C in all samples that was attributed mainly to ice melting (Vodovotz et al.,1996; Li, Dickinson and Chinachoti, 1998; Baik et al. 2001), was integrated and used to calculate the DSC frozen water content (FW). A decrease of frozen water content was observed during storage as already reported by other studies regarding bread staling (Baik et al. 2000, Hallberg et al. 2002; Ribotta and Bail 2007; Kerch, Rustichelli, Ausili, Zicans, Merijs and Glonin, 2008), resulting from the migration of a portion of water from gelatinized starch towards the more rigid amorphous and crystalline domains that so became unfreezable. FW decreased during storage from ~60 % (g frozen $H_2O$ / 100 g water) in fresh bread to ~44 % (g frozen $H_2O$ / 100 g water) in loaves stored at 25°C for 14 days (Figure C - 1C).

The endothermic peak in the temperature range 60-80°C found in all stored samples increased significantly during storage (Figure C - 1D) and indicated the melting of crystalline amylopectin as previously reported (Russell, 1983). DSC thermograms of stored samples exhibited also an endothermic event in the 50-80°C range (data not shown). In fresh samples no endothermic peak was observed in the 50-80 °C range and this indicate that the gelatinizeable starch in the bread dough had undergone the endothermic transition while cooking. In stored samples, the 50-80°C endothermic peak enthalpy increased more markedly at the early stage of storage and not significantly for storage longer than 3 days (Figure C - 1D).

Molecular characterization was carried out with multiple $^1H$ NMR experiments to cover a large range of molecular relaxation events. $^1H$ rotational mobility was studied, at 20 MHz, for the fastest-relaxing component, with a FID experiment while the slower relaxing proton fractions were characterized in terms of $T_2$ and $T_1$ relaxation times distributions. $^1H$ $T_1$ relaxation was also studied with FFC techniques over the 0.01 – 10 MHz frequency range. Translational $^1H$ molecular mobility was quantified in terms of the $^1H$ self diffusion coefficient.

$^1H$ FID decays are shown in Figure C - 2: the first, fast relaxing portion of the FID decay (<0,08 ms) is indicative of the presence of a very rigid $^1H$ population. It was observed that $^1H$ FID decays ($t < 0.01$ ms), representative of a more rigid component, became progressively more relevant during storage as already reported by other authors for gelatinized waxy maize starch (Farhat et al. 2003) and bread (Sereno et al. 2007). Such changes were attributed by the authors to a reduced mobility of the bread matrix due to both recrystallizing amylopectin and loss of water from the crumb.

The $^1H$ $T_2$ distributions obtained using an UPEN software were analyzed for $T_2 \geq 0.089$ ms (2 interpulse spacing + instrument dead time) to avoid extrapolation of $T_2$ values at times shorter than the first point measured with the CPMG experiment. $^1H$ $T_2$ quasi-continuous distributions are shown in Figure C - 3. Three $^1H$ $T_2$ populations were found and were named starting from the shorter to the longest relaxation time A, B and C, respectively. In fresh breads, $T_{2A}$ represented a population of protons characterized by relaxation times in the ~ 0.09 – 4 ms range and peaked at ~ 0.15 ms; the $T_{2B}$ protons relaxed in the ~ 6 – 20 ms range and peaked at ~ 10 ms; $T_{2C}$ protons were characterized by longer relaxation times (peaking at ~ 100 ms). Until 7 days of storage the $T_{2A}$ relaxation time did not undergo significant changes while both population B and C shifted towards shorter relaxation times. $T_{2B}$ relaxation time decreased significantly from ~12 ms to ~9 ms and $T_{2C}$ relaxation time

decreased from ~120 ms in fresh samples to ~111 ms during the 14 days of storage. $^1H$ $T_2$ The decrease in $T_{2B}$ peak during storage was previously reported in gelatinized waxy maize starch(Farhat et al., 2003) but other factors (such as gluten and/or water redistribution in the amorphous regions of the sample) may also play an important role (Hallberg et al. 2002; Vodovotz et al. 2002). A decrease of mobility of the more mobile protons ($T_{2C}$ relaxation time shifting towards shorter times) during storage was previously attributed to a decrease of overall proton mobility (Chen et al. 1997).

The relative amount of protons in each $^1H$ $T_2$ population was calculated (UPEN analysis, Borgia et al. 1998, Borgia et al. 2000) and the results were summarized in Figure C - 3 (right). $T_{2B}$ was the most abundant $^1H$ population encompassing 68.3 ± 1.2 % of the total protons in fresh samples while $T_{2A}$ represented 27.8 ± 0.9 % of total protons and $T_{2C}$ was the smallest $^1H$ population (< 4-5% of total protons). The relative amount of protons in population A and B changed significantly until 7 days of storage with A decreasing significantly to 21.9 ± 1.9 % (total protons), B increasing significantly to 73.3 ± 1.9 % (total protons). On the contrary the amount of protons of population C remained constant during storage.

The presence of multiple $^1H$ $T_2$ populations has been previously reported in baked products by several researchers. Our results are consistent with some previous studies: Engelsen et al. (2003) found three proton $T_2$ populations (with a 23.2 MHz spectrometer) peaking at ~0.5 ms, ~9-10 ms and ~21-30 ms that were attributed to water associated to protein, water associated to gelatinized starch (and pentosans) and diffusive exchange water between starch and protein, respectively. Wang, Choi and Kerr (2004) studied (with a 20 MHz spectrometer) some model systems (starch gels, gluten gels and starch-gluten gels) as well as bread samples to evaluate the effect of moisture content and gluten on proton mobility. They found two proton populations, peaking at ~0.1 ms and ~3.0 ms and attributed this last population to water associated with starch. Sereno et al. (2007) found one $^1H$ $T_2$ population peaking at ~9 ms (with a 23 MHz spectrometer) representative of the fast proton exchange between water and starch and the restricted water mobility within the polymers matrix. Chen et al. (1997) found three proton populations, peaking at 8-12 *μs*, 280-320 *μs* and 2.0-2.6 ms respectively and they attributed the shortest $T_2$ component to water associated to starch and gluten by hydrogen bonding. Also Ruan, Almaer, Huang, Perkins, Chen and Fulcher (1996) observed the presence of two proton populations in sweet rolls, peaking in the microseconds range and a second one peaking in the milliseconds range.

The three proton populations observed in bread loaves were, therefore, tentatively assigned to protons associated to water-protein phase (population A), to protons associated with the gelatinized starch phase (population B) and more mobile, exchanging protons (population C). The decrease of the amount of protons belonging to population A and the corresponding increase of the protons in population B might indicate a migration of water from the gluten domain to the starch domain during storage.

At longer storage (14 days), two population were found: a broader proton population (AB), characterized by two peak times ($T_{2AB1}$ ~0.15 ms and $T_{2AB2}$ ~7 ms), encompassing ~95% of the total protons and a smaller population (C, $T_{2C}$ ~111 ms). It is likely that the $^1H$ population AB resulted from the overlapping of the A and B proton populations that at longer storage times were no longer resolved (clearly separated) as the protons underwent exchange within the NMR experimental time-frame.

Proton $T_1$ distributions of bread crumb during storage are shown in Figure C - 4 for selected frequencies (0.01, 0.52, 10 and 20 MHz). All $^1H$ $T_1$ distributions were unimodal (with the exception of the distribution acquired at 0.52 MHz) and the representative $^1H$ $T_1$ relaxation times decreased from ~ 100 to ~ 7 ms with frequency decreasing from 20 to 0.01 MHz in fresh breads. The representative major peak of $^1H$ $T_1$ relaxation times distributions significantly shifted towards shorter relaxation times at all frequencies during storage. The $^1H$ $T_1$ relaxation time reduction during storage is better represented by a plot of $^1H$ $R_1$ (= $1/^1H$ $T_1$) as function of frequency (Figure C - 5). The relaxation rates (= $1/^1H$ $T_1$) increased from ~ 9 $s^{-1}$ to ~ 11 $s^{-1}$ at 20MHz, from ~ 68 $s^{-1}$ to ~ 70 $s^{-1}$ at 0.2MHz, from ~ 87 $s^{-1}$ to ~ 92 $s^{-1}$ at 0.07MHz, from ~110 $s^{-1}$ to ~ 121 $s^{-1}$ at 0.03MHz and from ~ 125 $s^{-1}$ to ~ 144 $s^{-1}$ at 0.01MHz. The mobility loss was more marked at frequencies lower than 0.2 MHz. In particular, $^1H$ $T_1$ distributions of relaxation times acquired at 0.52MHz showed a second proton population relaxing at ~2 ms and encompassing ~ 7% of the total protons until 3 days of storage, indicating the presence of less mobile protons that was not detectable at longer storage.

Considering the space-time frame investigated at lower frequencies (0.52MHz), the protons belonging to the above-mentioned population could be related to a water-macromolecules domain. It may be speculated that these protons are associated to the water-gluten phase, since they can be representative of motions of large macromolecule systems . Their mobility decrease caused them not to be detectable in the time-space domain of the experiment,

standing for the partial dehydration and plasticization loss the gluten underwent during storage.

The self diffusion coefficient (D) is representative of translational motions of water protons. $^{1}H$ self diffusion coefficients of bread loaves during storage are shown in Figure C - 6. The observed results are consistent with previous studies on wheat starch gels (~0.5 * $10^{-9}$ $m^2*s^{-1}$ 0.67 g of water/ g of solids at 25°C; Gomi, Fukuoka, Mihori and Watanabe, 1998) and starch-gluten-water mixtures (~0.2-0.6 * $10^{-9}$ $m^2*s^{-1}$ at 0.54 - 1.00 g of water/g of solids at 30°C; Umbach, Davis, Gordon and Callaghan, 1992) but they are not comparable to those found by Baik and Chinachoti (2003) in white bread (0.067 * $10^{-9}$ $m^2*s^{-1}$ at 35°C). Significant changes were detected over storage but they were not associable to water state and dynamics, as previously reported (Baik and Chinachoti, 2003)

## 5. Conclusions

Bread staling of white bread was analyzed over a wide range of time-space domains ranging from molecular (low resolution NMR spectroscopy) to macroscopic (crumb hardness). $^{1}H$ NMR FFC technique was used to investigate molecular mobility ($^{1}H$ $T_1$) at low frequencies (0.01 - 20MHZ) for the first time on bread.

The investigation of bread staling confirmed the previous related works in terms of macroscopic (texture analysis, moisture content) and macromolecular (frozen water content and amylopectin recrystallization) properties. Proton molecular mobility was found to be a good indicator of bread staling phenomena: molecular mobility changes were detected and the results were consistent with those reported by previous studies. $^{1}H$ NMR FFC allowed to investigate protons $T_1$ relaxation at frequencies lower than 20MHz. Few studies reported $^{1}H$ $T_1$ mobility results regarding bread staling and it was not reported about $T_1$ mobility changes attributed to specific molecular phenomena occurring during bread staling. A new insight of $T_1$ relaxation process was achieved by means of Fast Field Cycling $^{1}H$ NMR that underlined a more marked $T_1$ mobility loss at frequencies lower than 0.2MHz. In particular, the presence of two protons population at 0.52MHz was tentatively attributed to changes related to the macromolecular (gluten) domain.

## 6. List of figures

**Figure C - 1**:

**A:** Bread crumb (•) and crust ( ) moisture content during storage
**B:** Bread crumb hardness during storage
**C:** DSC Frozen water content of bread crumb during storage
**D:** DSC Amylopectin melting in bread crumb during storage

Symbols with the same letter do not significantly differ ( $p \leq 0.05$)

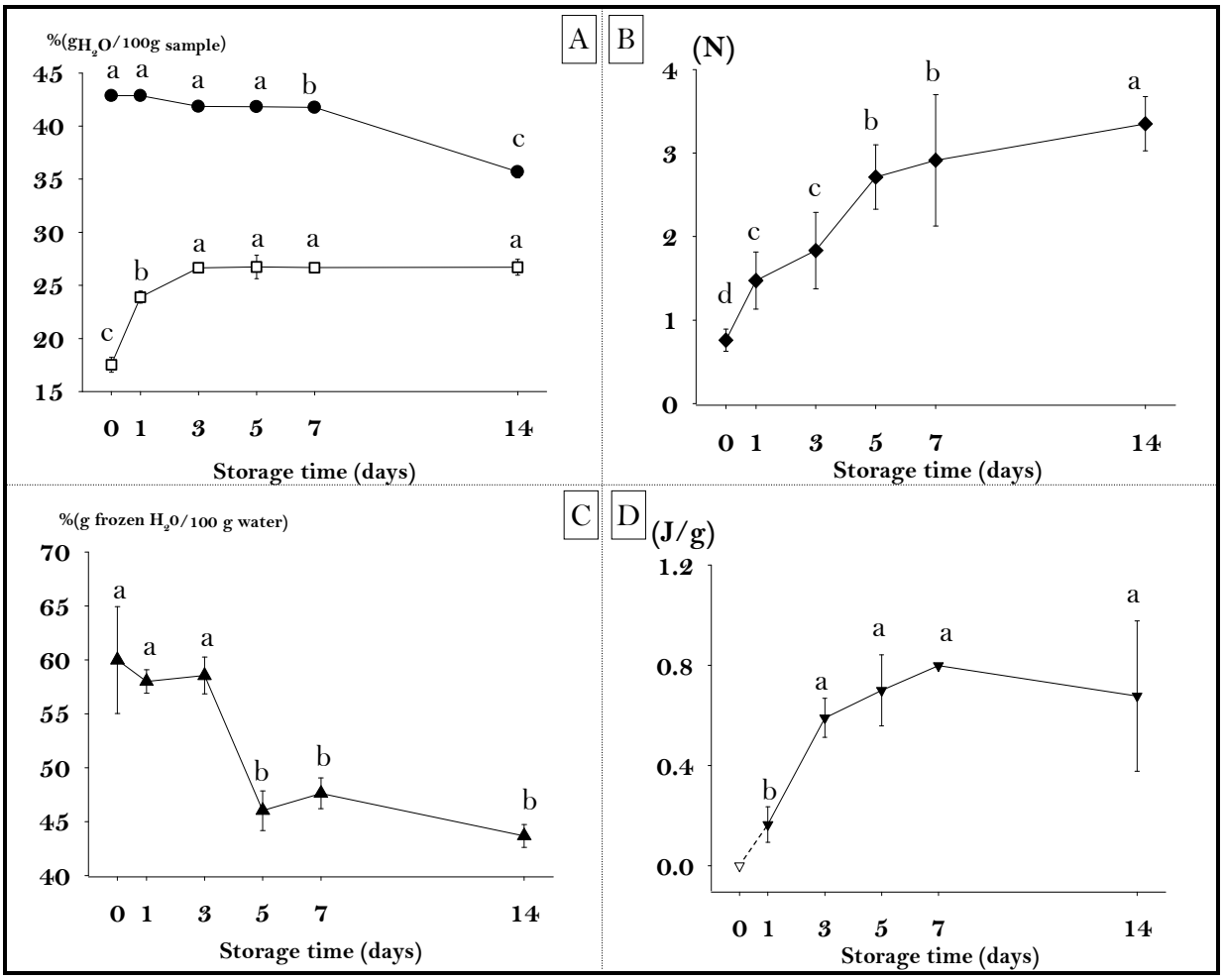


**Figure C - 2**: $^{1}H$ FID decays of bread crumb during storage

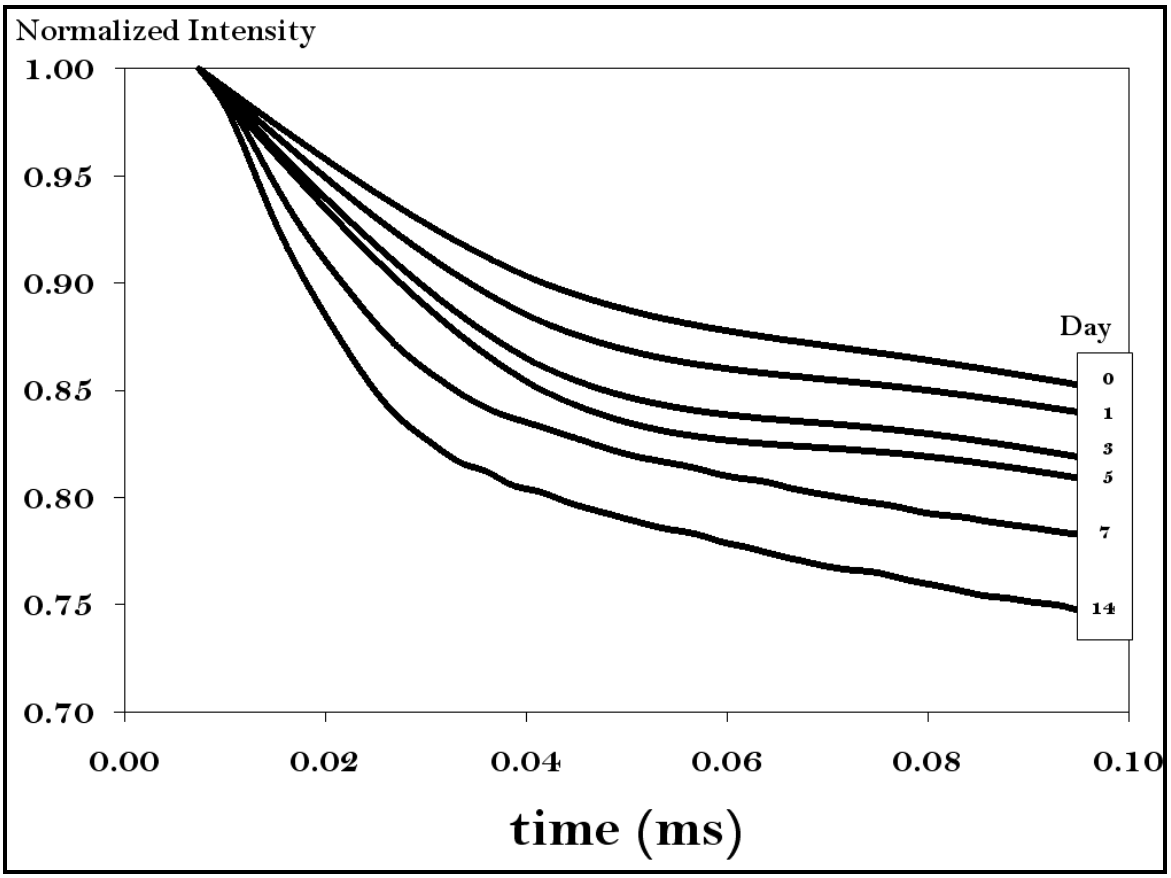


**Figure C - 3**: $^{1}H$ $T_2$ relaxation times distributions (left) of bread crumb during storage and relative abundance of the proton populations (right).

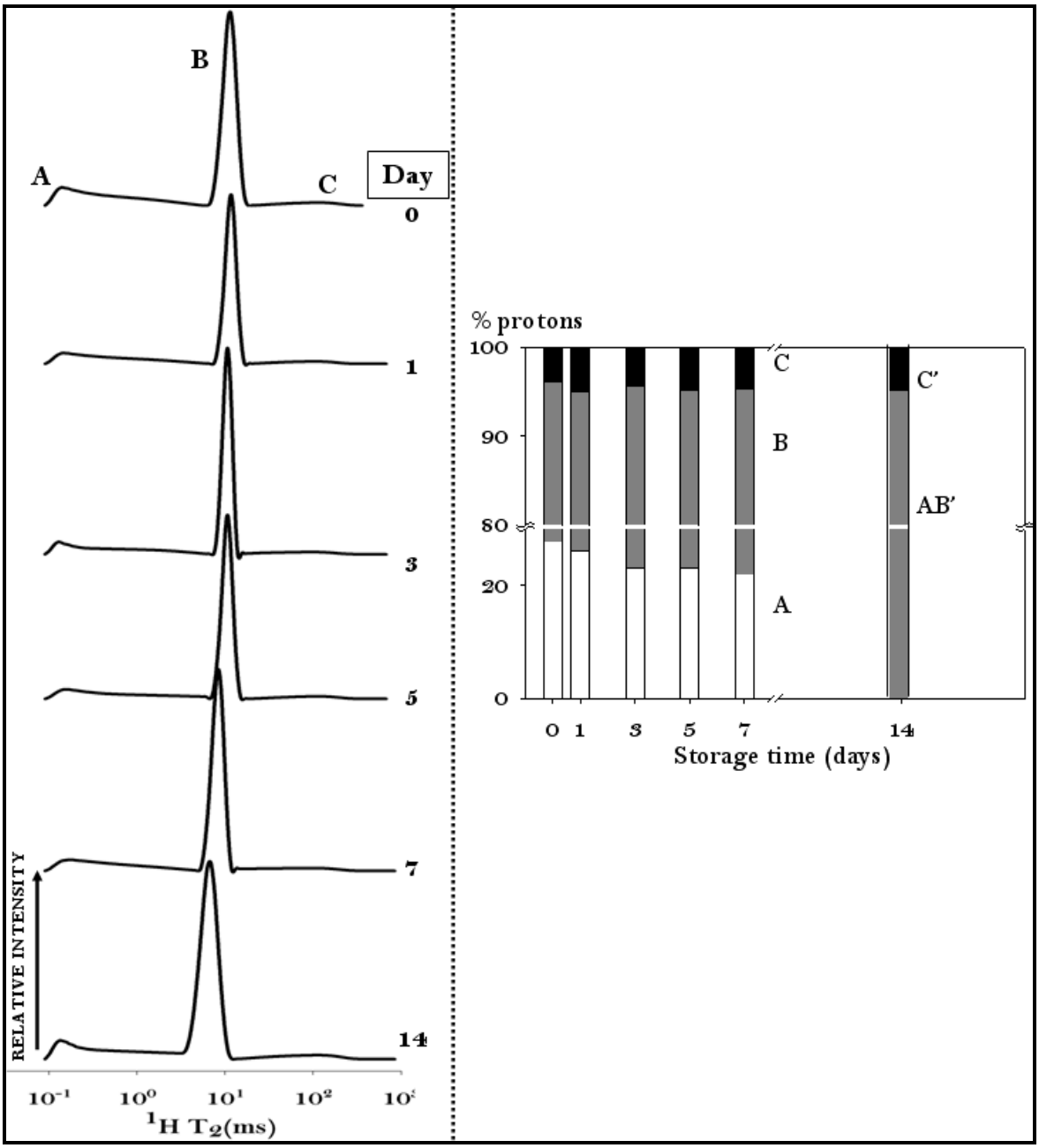


**Figure C - 4**: $^{1}H$ $T_1$ relaxation times distributions at 0.01, 0.52, 10 and 20MHz during storage

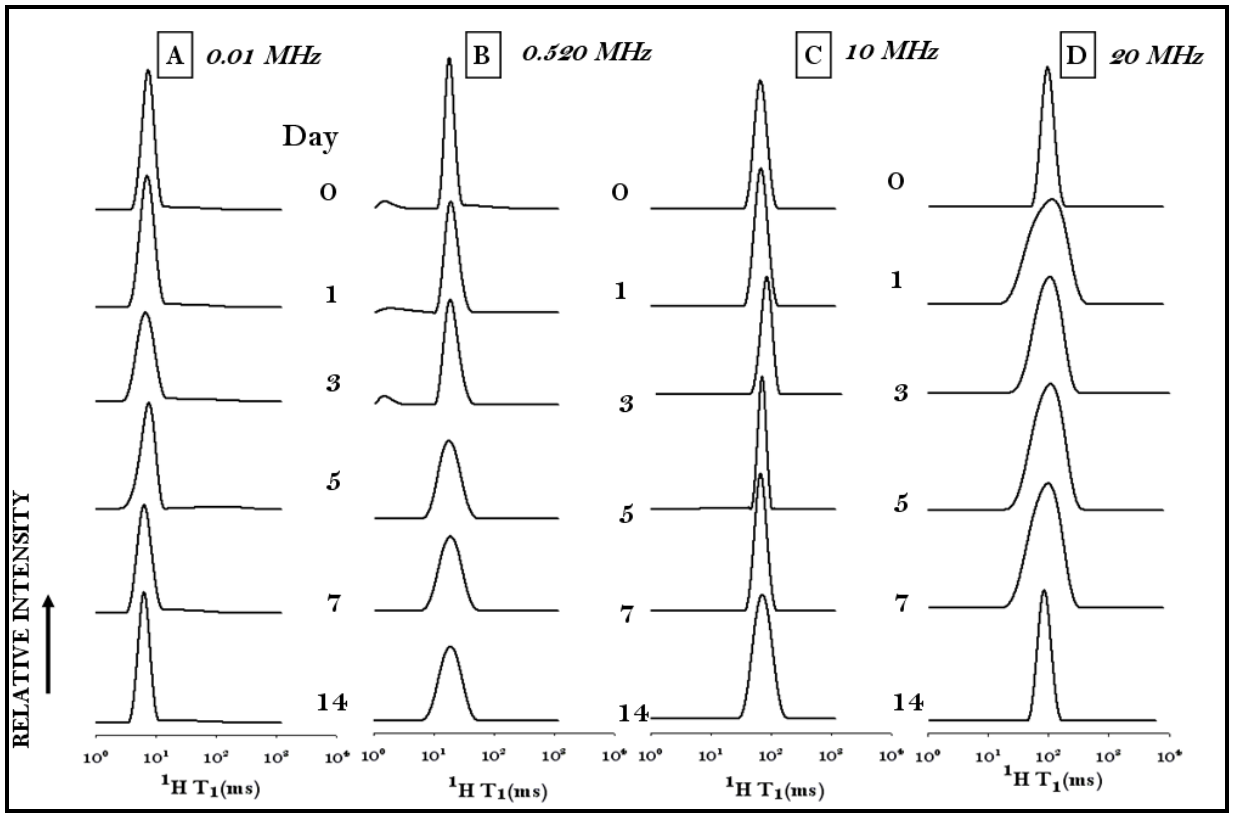


**Figure C - 5**: $^1T_1$ NMRD profile of bread crumb samples during storage at all FFC frequencies (A) and at lower frequencies (B)

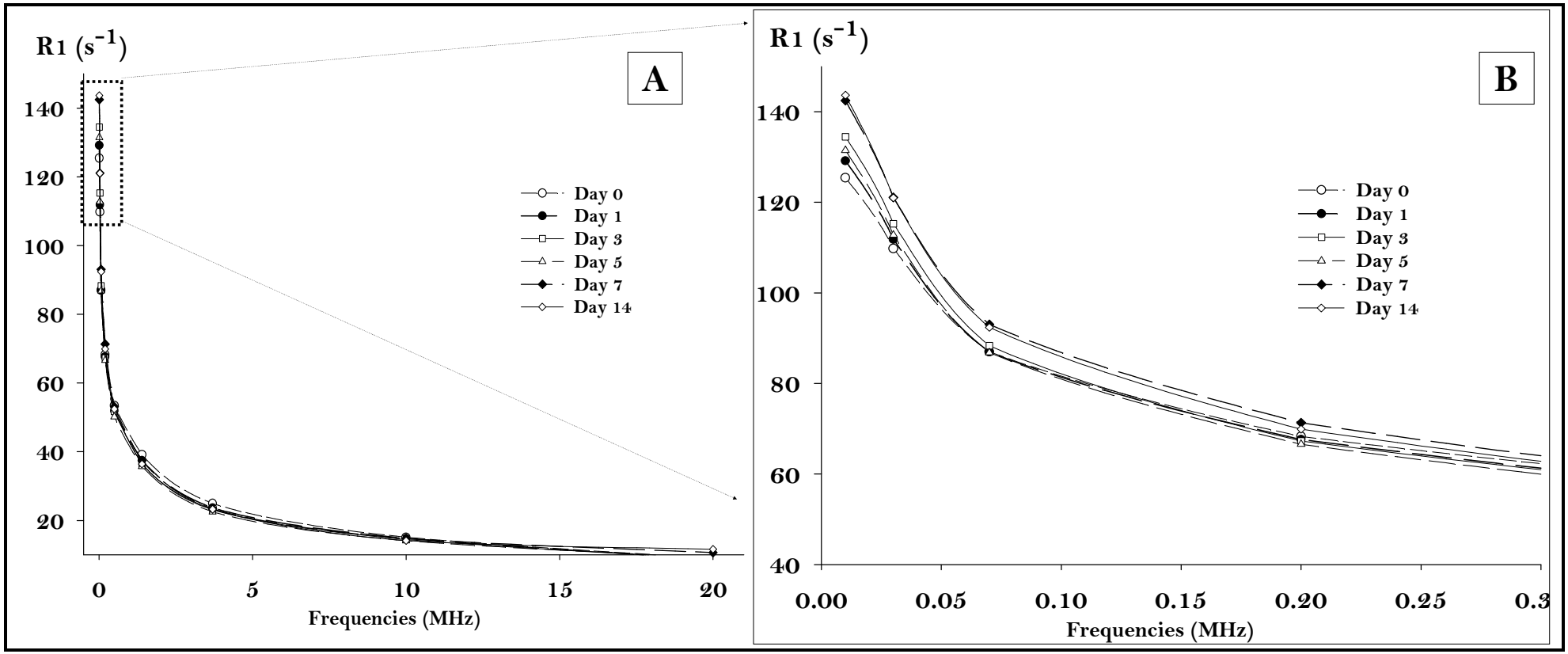
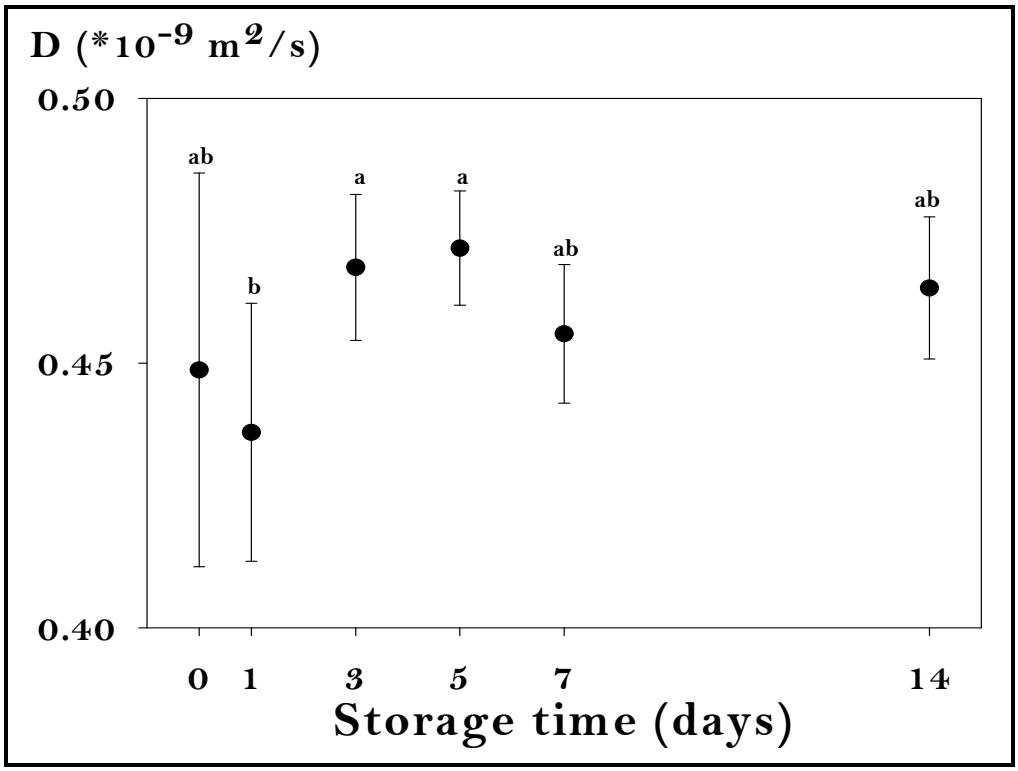


**Figure C - 6**: $^{1}H$ Self diffusion coefficient during storage
Symbols with the same letter do not significantly differ ( $p \leq 0.05$)



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## Publications

- Carini, E., Curti E., Vittadini, E. "Effect of long term storage on water redistribution and physico-chemical properties of nutritionally enhanced tortillas" Food Biophysics, submitted.
- Carini E., Vittadini E., Curti E., Antoniazzi F. "Physicochemical properties and water status of fresh pasta produced with different shaping modes", Journal of Food Engineering, 93 400–406 (2009).
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