Water status and dynamics of high-moisture Mozzarella cheese as affected by frozen and refrigerated storage

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Abstract: High-moisture Mozzarella is one of the most exported cheeses worldwide, but affected by short shelf-life. Freezing can help to reduce waste, but its effect on quality needs to be considered. In this study, the physico-chemical changes of Mozzarella occurring during frozen storage and subsequent refrigerated storage (after thawing) were evaluated. Frozen cheeses stored at -18°C between 1 and 4 months showed microstructural damage and different physical, textural, sensory properties. With NMR relaxometry it was possible to observe freeze-related dehydration of caseins, by measuring the changes in water relaxation times within the matrix. These modifications were confirmed by microstructural observations that showed the formation of larger serum channels in samples subjected to freezing, compared with fresh cheeses. Sensory evaluation showed skin peeling off in frozen samples. By observing the changes at various length scales it was therefore possible to identify the critical points affecting HM Mozzarella cheese quality during frozen storage.

*Cover Letter

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Prof. A. Sant'Ana
Editor-in-Chief
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May 22, 2020

Dear Prof. Sant'Ana,

On behalf of all authors, I would like to re submit the revised research article entitled "Water status and dynamics of high-moisture Mozzarella cheese as affected by frozen and refrigerated storage" by Marcello Alinovi, Milena Corredig, Germano Mucchetti, and Eleonora Carini for consideration for publication in *Food Research International.*

We appreciate the interest that the Editor and Reviewers have taken in our manuscript and the constructive criticism they have given. We hope to have addressed the major observations of the Reviewers and Editor. In particular, we improved language quality and we tried to clarify NMR section, by following Reviewers' suggestions and comments. We feel that these changes have clearly improved our manuscript. We have also included a point-by-point response to the Reviewers in addition to making the changes described above in the manuscript. Changes to the manuscript are formatted as "Tracked Changes".

Our main findings remain unchanged. NMR relaxometry together with microstructural, textural and sensory observations, was able to highlight water status changes in high-moisture Mozzarella cheese as affected by frozen storage and freeze-related dehydration of caseins.
Considering the increasing global demand for high-moisture traditional cheeses such as high-moisture Italian Mozzarella cheese, we believe that this manuscript is appropriate for publication by *Food Research International* as it improves knowledge about the detrimental phenomena that affect Mozzarella cheese frozen storage and could be a starting point to improve frozen Mozzarella cheese quality characteristics; moreover, it highlights the potentiality of NMR relaxometry to be an analytical tool useful to investigate water related changes in Mozzarella cheese.
This manuscript has not been published and is not under consideration for publication elsewhere and we have no conflicts of interest to disclose. Please address all correspondence concerning this manuscript to me at marcello.alinovi@studenti.unipr.it.

Thank you for your consideration.

Sincerely yours,

Marcello Alinovi

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*Detailed Response to Reviewers

Response to reviewers

Manuscript FOODRES-D-20-00954 entitled

Water status and dynamics of high-moisture Mozzarella cheese as affected by frozen and refrigerated storage

We would like to thank the Reviewers and Editor for their time and efforts in reviewing this manuscript, and for the constructive feedback which they were able to provide. We feel these suggestions have helped to substantially improve the quality of this manuscript. Each comment has been carefully considered point-by-point. In addition, with Reviewers suggestions, we revised Figure 5 because of a decimal separator issue in panels A and B. Our responses to the Editor and Reviewers are below (responses to Reviewers comments in red) and changes made in the manuscript are highlighted using "Tracked Changes" option.

Reviewer #1: The authors studied the water status and dynamics of high-moisture Mozzarella cheese as affected by frozen and refrigerated storage. Here are the comments:

1. The manuscript is not easy to follow, in the section of experimental design, some details should be more specific: the total number of samples and the number of samples in each different state for analysis. Were all the frozen-thawed samples refrigerated for 1 day, and then 3 days, and then 8 days or just divided into 3 groups and refrigerated for 1,3 and 8 days respectively? And how many fresh non-frozen cheese samples (normal control group) were analyzed or just analyzed the same samples before being frozen? So, the order of the description should be considered to avoid confusion if all the samples were analyzed in fresh state and then different frozen and refrigerated states.

We acknowledge the Reviewer for this comment. Accordingly, we decided to improve clarity and readability of the experimental design section. We also added a schematic representation of the experimental design (Figure 1).

In this experimentation, each sampling group was independent: this mean that each cheese (sampling unit) was only directed to one treatment and analyzed once. Accordingly, control, non-frozen cheeses were analyzed only at 1, 3, 8 days of refrigerated storage and were not frozen.

According to the complete block design experimental design, each batch of Mozzarella cheeses (blocking factor of the design, corresponding to the technological replication of the matrix) was divided into 4 main groups (main plot of the design), corresponding to the control and the 3 frozen stored groups (1, 3, 4 month of storage). Each of these main groups was further divided into subgroups (corresponding to the sub plots of the design), each one representing a different refrigerated storage time (1, 3, 8 days of storage). In this kind of design, the single cheese represents a sampling unit of the group.

2. In the section of statistical analysis, what's the purpose of using this split-plot ANOVA models? And the which result part shows the significance of frozen storage and refrigerated storage interactions?

We acknowledge the Reviewer for this comment. From previous research works, we were aware that product's properties could vary within cheese batches as a consequence of differences in terms of milk characteristics and of manufacture parameters of the industrial cheesemaking process. For this reason, in order to limit the influence of batch-to-batch variations, a blocked-type factorial design of experiment was used, as reported in the Experimental design section.

With this type of design, it is possible to take into account the degree of variability given by these random variations by considering the batch of cheese as the blocking factor of the design; this kind of design is used to avoid false detection of significant effects of evaluated variables that can be caused by a nuisance variation of the factor to be blocked (Tsai, 2016).

Accordingly, the statistical analysis was performed by considering the batch of cheese as the nuisance factor. A split-plot ANOVA model was considered. This statistical model combines the blocking design of experiment together with the fractional factorial organization (split-plot) and it is advantageous with the simultaneous presence of a hard-to-change factor (in this case, frozen storage time) and a nuisance factor (McLeod & Brewster, 2004).

The significant interaction between the two main factors frozen storage and refrigerated storage can be viewed from tables S1 and S2 reported in supplementary material. The P-value associated to the term *Ft* × *Rt* determine the statistical significance of this interaction.

McLeod, R. G., & Brewster, J. F. (2004). The design of blocked fractional factorial split-plot experiments. Technometrics, 46(2), 135–146. https://doi.org/10.1198/004017004000000176

Tsai, P. W. (2016). A study of two types of split-plot designs. Journal of Quality Technology, 48(1), 44–53. https://doi.org/10.1080/00224065.2016.11918150

3. In NMR results part, line 315: "In our study, changes shown in T1 relaxation times and relative abundance of proton populations can not be attributed to protein hydration…" Line 301: "This migration of water is related to freeze-induced protein dehydration and related to the increase of ES during the frozen storage; in particular the increase of component B was significantly related to the increase of ES (r=0.380)." These two descriptions may cause confusion. It may be better to merge these two parts into one part to explain the relationship between T1 relaxation changes and protein dehydration. The whole section of NMR results should be reorganized to show the result and analysis more clearly and directly with the help of figures or tables.

We acknowledge the Reviewer for this comment. As suggested, we merged the sentence present at line 301 of the old manuscript with the sentence at line 315 of the old manuscript. The revised sentence now is (line XXX of the revised manuscript): “In our study, changes shown in T_1 relaxation times and relative abundance of proton populations can not be attributed to protein hydration, as observed during the refrigerated storage of LM Mozzarella cheeses (Guo & Kindstedt, 1995; Kuo & Gunasekaran, 2009; Kuo et al., 2001); there was a different behavior, confirmed by both NMR and ES measurements, caused by freezing. In particular, the migration of water from population A to population B can be related to freeze-induced protein dehydration and related to the increase of ES during the frozen storage; the increase of component B was significantly related to the increase of ES (r=0.380). As freezing can promote modification of the tertiary and quaternary structure of proteins (Xiong, 1997), it may lead to irreversible dehydration of the protein matrix.

Moreover, according to the Reviewer comment, further revisions were reported on the whole NMR results section, in order to improve clarity and readability.

4.In NMR results part, line 341: "In our study, it was not possible to make a clear distinction between the two components, as they were largely overlapped (Figure 3)." As described in this part, those peaks were largely overlapped, so how to calculate the proportion of component B and C in Figure 4 (c) and (d)?

We acknowledge the Reviewer for this question.

The fitting of NMR data can be performed with different approaches. The available approaches are based on different algorithms that provide different outputs and degree of information.

A first approach can be the use of a software (i.e. Upen, Contin) able to invert the decay data in order to give quasi-continuous distributions of relaxation times, so one can observe how many protons populations relax in the experimental time-frame window (i.e. proton T_1 and proton T_2 experimental time window). With this approach, it can also be observed if some protons populations are overlapped with others and therefore discuss the possible inhomogeneity of protons exchange between different protons domains (i.e. protons populations). In this case, an average relaxation time and an overall relative protons abundance related to the peak generated by the two or more overlapped populations is given back by the software, but no information about the relaxation time and relative abundance of the single observed population can be obtained.

A second approach is to fit the experimental curves with multi-components exponential model equation. In this case, the number of components of the model are in most cases arbitrary chosen based on information taken by the relative literature or previous experimental data. With this approach, one can obtain quantitative information concerning relaxation times and relative abundances of populations which, as we reported before, are pre-determined in an arbitrary way. Thus, this approach is more informative than the first approach, but in some cases could be misleading with respect to the "real" situation of the sample (i.e. different number of populations).

A third approach could be the use of both the above-mentioned methods. In fact, based on the number of protons populations found by applying a software that inverts the decay data to give quasi-continuous distributions of relaxation times, one can further apply a discrete multi-exponential model (the number of components are decided as the number of protons population observed with the quasi-continuous model and/or in accordance with previous literature findings) to determine the exact contribution of all the protons populations (in terms of relaxation time and relative abundance), including those that are partially overlapped. In this manner, additional information can be obtained and used to found possible correlation with other properties. This approach could be very useful when the experimental design includes the use of different techniques to study different food properties. Indeed, the relation of molecular information derived from NMR to information probed at different structural levels is a matter of particular interest. To be able to do this, a punctual determination of relaxation times and relative abundances of all detected NMR protons populations is fundamental. However, also this approach is not free to limitation, since determining the relaxation times and relative abundance of protons populations including those that are partially overlapped means considering the system as more homogeneous than it is from a molecular point of view.

In this study, we used this third approach applying the UPEN software to invert the decay data and obtain the quasi-continuous distribution of relaxation times and to determine the number of protons populations relaxing in the ^1H T_1 and T_2 relaxation time windows and successively, we used a discrete multi-exponential model to obtain relaxation times and relative abundances of all populations which were used in the statistical model of the study to find correlation with other properties (as it has been reported in the discussion). In our opinion and in this case, this is the best approach of the available ones and it has been used in other previous works when a multiscale study was performed in various food matrices*.

We acknowledge that the methodology used in this study was not completely clear and explained in the Material and Methods section. In particular, the use of UPEN software has not been reported. For this reason, thanks to the Reviewer comment, we implemented section 2.4 (L194-199) by reporting the use of UPEN software to obtain the quasi-continuous distributions of relaxation times of ^1H T_2 T_1 curves.

According the Material and Methods section 2.4, by fitting T_2 relaxation curves with discrete multiexponential models, it was possible to estimate the relative intensity of each component (A_2 (i)). By dividing the relative intensity of each population *i* for the intersect (L_2) of the polynomial function that represent the instrumental noise of the measurement, it was possible to estimate the abundance (expressed in relative percentage) of each population.

*

- *Diantom et al., 2020, Can potato fiber efficiently substitute xanthan gum in modulating chemical properties of tomato products?, Food Hydrocolloids, 101, 105508*
- *Littardi et al., 2020, Quality evaluation of chestnut flour addition on fresh pasta. LWT, 109303*
- *Diantom et al., 2019, A multi-scale approach for pasta quality features assessment, LWT, 101, 285-292*
- *Diantom et al., 2017, Effect of added ingredients on water status and physico-chemical properties of tomato sauce, Food Chemistry, 236, 101-108*
- *Carini et al., 2017, Staling of gluten-free breads: physico-chemical properties and 1 H NMR mobility, European Food Research and Technology, 243(5), 867-877*
- *Curti et al., 2017, Staling and water dynamics in high-gluten bread, European Food Research and Technology, 243(7), 1173-118*
- *Curti et al., 2017, The use of two-dimensional NMR relaxometry in bread staling: a valuable tool?, Food Chemistry, 237, 766-772*
- *Diantom, et al., 2016, Effect of water and gluten on physico-chemical properties and stability of ready to eat shelf-stable pasta, Food Chemistry, 195, 91-96*

5. In NMR results part, figure 3 only shows the T2 relaxation of the cheese refrigerated for 8 days. How about T2 relaxation of cheese refrigerated for 1 day and 3 days? The description of figure 4 (b) and (d) doesn't show which frozen storage the cheeses were.

Thank you for your comment. The figure 3 of the old manuscript (now figure 4) is meant to be a simple and most immediate visual representation of the changes in ^1H T_2 populations involved during refrigerated and/or frozen storage. For this reason, we decided to show only a part of the samples. In this figure, control at 1 and 8 days of refrigerated storage, and frozen thawed samples at 1, 3, 4 months of frozen storage and at 8 days of refrigerated storage are showed in order to give to the reader a brief overlook of the changes of protons populations encountered during this study. Accordingly, not only control sample at 3 days of refrigerated storage, but also frozen thawed samples at 0 and 3 days of refrigerated storage are lacking.

However, as significant changes in frozen and refrigerated storage are deeply illustrated in figure 4, we decided to keep this figure as it is.

Concerning Figure 4, all the graphs were built by averaging the data as a function of all the refrigerated storage times (in the case of panel a, c) and of all the frozen storage times (in the case of panel b, d). Thanks to Reviewer comment, we added this information into the figure caption.

This data representation is usually performed in the case of split plot ANOVA models in order to represent the significant effect of one main factor (Ft or Rt). As in those cases, when in a factorial model one or both main factor are significant, but the interaction is not (please have a look at Table S2), the data representation is done by grouping the data only for the significant(s) factor(s), and not by subdividing the representation of the dataset for all the sub groups (e.g.: Rt=1 and Ft=0, Rt=1 and Ft=1, Rt=1 and Ft=3, Rt=1 and Ft=4, Rt=3 and Ft=0, and so on..).

6. The overlapping of peaks in NMR relaxation spectrum can be adjusted by using different regularization parameter (or so-called smooth factor) in inversion method. The selection of regularization parameter depends on the noise level of NMR signal (obtained by using IR\SR\CPMG pulse sequence). So, what's the SNR of the cheese NMR signal?

The SNR was 650 ± 128.

What's the smooth factor of your relaxation spectrum?

The smoothing factor was 1 that is software worked in an over smoothing manner, as this parameter resulted always <2.

And how many points in your relaxation spectrum? These parameters may be optimized to get a better relaxation spectrum to separate those peaks.

Number of points of the relaxation spectrum were 100.

Additional details about the adjustments that software is able to implement can be found in these two references where authors explain in a detailed way the software specifications (Borgia, G.C., Brown, R.J.S., Fantazzini, P., 1998. Uniform-penalty inversion of multiexponential data decay. Journal of Magnetic Resonance 132, 65–77; Borgia, G.C., Brown, R.J.S., Fantazzini, P., 2000. Uniform-penalty inversion of multiexponential data decay II. Data spacing, T2 data, systematic data errors, and diagnostic. Journal of Magnetic Resonance 147, 273–285).

If the reviewer has particular suggestions about the regularization of the relaxation spectrum in order to improve NMR signals authors will particularly be grate for this.

7、There are some obvious errors in singular and plural expression, phrase usage and so on. I suggest the authors should carefully read and check them before re-submission.

For example, "…showed the formation of larger serum channels in samples subjected to freezing, 'compare with' fresh Mozzarella." (at line 31 of page 2)

"microstructural damages" at line 28 of page 2.

Thank you for pointing it out. The manuscript has been revised by a native speaker. We double checked the whole manuscript and we corrected this kind of grammar error, plus other misspelled or incorrect terms.

8、To the knowledges of the reviewer, "T1 relaxation time", also called "longitudinal relaxation time", "spin-lattice relaxation time", but "T1 spin-lattice longitudinal relaxation times" seems not to be correct. (at line 180, 184 of page 8)

We acknowledge the Reviewer for pointing this out. We corrected it as “spin-lattice relaxation time”. Accordingly, we modified the statement at L191 of the revised manuscript (“spin-spin relaxation time”).

9、At line 283 of page12, "relaxation component" is suggested to replace "relaxing component".

Thank you. As suggested by the Reviewer, we changed “relaxing component” into “relaxation component”.

10、For equation (2), the exponential decay signal obtained by inversion-recovery pulse method is

Unfortunately, the Reviewer question/comment was truncated. Despite of this, the comment highlighted a miscopy in the equation 2, that has been corrected.

The corrected equation 2 reported in the revised manuscript is:

$$M(t) = \sum_i M_{\infty(i)}(1 - 2e^{-\tau/T_{1(i)}}) \quad (2)$$

11、In 3.2 1H T1,T2 NMR results section, the Figure 3 seems to have only two peaks, please explain the reason of four components, why not two or three components.

Thank you for your question. We agree with the Reviewer. However, by the observation of the proton distribution of relaxation times, it can be noticed the presence of three protons populations (B, C and D) contributing to the broad slower population. For this reason, and based on reasoning behind the choice of the type of approach used for the data fitting (explained in the response of point 4 to the Reviewer), we decided to fit T_2 relaxation curve by also using a discrete multiexponential polynomial function having 4 terms. Moreover, this way of interpreting T_2 relaxation data is in accordance (and then, it can be compared) with previous findings reported in the case of Buffalo Mozzarella cheese (Gianferri et al., 2007 a & b).

Gianferri, R., D'Aiuto, V., Curini, R., Delfini, M., & Brosio, E. (2007). Proton NMR transverse relaxation measurements to study water dynamic states and age-related changes in Mozzarella di Bufala Campana cheese. Food Chemistry, 105(2), 720–726. https://doi.org/10.1016/j.foodchem.2007.01.005

Gianferri, R., Maioli, M., Delfini, M., & Brosio, E. (2007). A low-resolution and high-resolution nuclear magnetic resonance integrated approach to investigate the physical structure and metabolic profile of Mozzarella di Bufala Campana cheese. International Dairy Journal, 17(2), 167–176. https://doi.org/10.1016/j.idairyj.2006.02.006

12、In 3.2 1H T1,T2 NMR results section， the major components(B,C,D) in the T2 curves (Figure 3) are partially overlapped. Please explain how to accurately calculate the population or relative population of component B and C (Figure 4).

Thank you for your question. Please, have a look at answer to question n.4.

13、At line 327 of page 13, "at very short relaxation times (~0.1-1ms) " is suggested. The relaxation time of component A is not located at ~1-2ms.

Thank you for pointing this out. We specified the maximum of population A, that as suggested is in between 0.1-1 ms and we reported also the distribution range. In particular, component A relaxed in the range between 0.1 and 4.2 ms and showed the peak at ~ 0.1-0.2 ms.

Moreover, we would point out that population A, as observable from the quasi-continuous distributions of relaxation times obtained using UPEN was characterized by a highly non normal distribution.

For this reason, the relaxation time corresponding to the maximum peak observable from the proton distributions in Figure 4 of the revised manuscript, was not coincident with the relaxation time estimated using the discrete multi-exponential model with Sigmaplot. When in presence of highly non gaussian

proton distributions, the relaxation time obtained with these two different methods can be slightly divergent, although the relaxation range was comparable.

14、 As three different types of water in chesses were introduced in introduction part, what are those peaks (A, B, C and D) in T2 relaxation spectrum mean?

Thank you for your question. As reported in the Results and Discussion section 3.2, component A may correspond to the ^{1}H of water strongly bound to the casein structure as solvation water, component B and/or C can be related to protons of water trapped in the protein meshes, while component D is related to a fraction of the physically trapped water population, that can be located into serum channels and is weakly physically kept by the matrix. It is important to note that also protons associated with the fat phase can be detected by T_2 analyses and can be attributed to component B and/or C. This description of proton populations is consistent with that reported by Gianferri et al., 2007a,b. Moreover, we feel that it is also in accordance with that reported in the introduction section, as the presence of an additional proton population is not caused by the water phase but by the fat phase.

Gianferri, R., D'Aiuto, V., Curini, R., Delfini, M., & Brosio, E. (2007). Proton NMR transverse relaxation measurements to study water dynamic states and age-related changes in Mozzarella di Bufala Campana cheese. Food Chemistry, 105(2), 720–726. https://doi.org/10.1016/j.foodchem.2007.01.005

Gianferri, R., Maioli, M., Delfini, M., & Brosio, E. (2007). A low-resolution and high-resolution nuclear magnetic resonance integrated approach to investigate the physical structure and metabolic profile of Mozzarella di Bufala Campana cheese. International Dairy Journal, 17(2), 167–176. https://doi.org/10.1016/j.idairyj.2006.02.006

15、 Figure 8 shows the result of PCA, it seems that there were only 9 points of each group in figure 8(a) and 12 points in figure8(b), not consistent with the description of line 126: "groups of fifteen cheeses for each batch were kept in frozen storage for 1, 3, and 4 months and then thawed."

Thank you for your comment. According to experimental design, each point in the PCA graph represented a cheese group (that correspond to a single sample, in the statistical design) identified as the combination of factors reported in the experimental design (batch of cheese, frozen storage time, refrigerated storage time). According to this grouping, a total of 36 groups can be identified (3 cheese batches x 4 frozen storage times x 3 refrigerated storage times), corresponding to the points represented in the PCA's score plots. Accordingly, if you classify the score plot according to the frozen storage times, each frozen storage group will have a numerosity of 9 (corresponding to the total number of groups or samples (36), divided the number of frozen storage treatments (4)). In the same way, if you classify the score plot according to the refrigerated storage times, each refrigerated storage group will have a numerosity of 12 (corresponding to the total number of groups or samples (36), divided the number of frozen storage treatments (3)).

Reviewer #2: Very well written and very well structured manuscript. It was understandable and easy to read and follow and gives valuable data about the frozen storage of high moisture mozzarella cheese taking

into the consideration the changes in water (moisture) content, microstructure, texture and sensory perception. I recommend it to be accepted as it is and the only remark is to check the % in Line 282.

Thank you for your comment. We checked and corrected the punctuation.

Reviewer #3: This manuscript is dealt on water status and dynamics of high-moisture Mozzarella cheese affected by frozen and refrigerated storage. The freezing phenomenon of cheese was well explained. This paper is well organized and clearly written. However, this manuscript have demanded some minor revision.

Line 78, 80, 102, 107 Delete "," Alinovi & Mucchetti (2020b); Conte et al. (2017); …

Thank you, we corrected the manuscript according to this suggestion.

Please indicate the size (diameter) of the mozzarella cheese in Line 138.

Thank you, we added this information in the manuscript at lines 142-144:" as each cheese was characterized by a non-regular spheroidal shape (Alinovi & Mucchetti, 2020a), it was characterized by a variable diameter of 4-6 cm."

Line 150 Deleted bracket

Thank you, we added the left bracket.

Table 1. Physical, chemical parameters as a function of the different frozen storage times (

fresh cheese, 1, 3, 4 months of frozen storage).

Thank you, we corrected the caption according to Reviewer comment.

Table 2. Textural parameters as a function of the different frozen storage times (fresh cheese, 1, 3, 4 months of frozen storage).

Thank you, we corrected the caption according to Reviewer comment.

Figure 5 (a) bracket (c) 3 months

Thank you. We corrected it according to this suggestion.

Figure 8 = 3 months

Thank you. We corrected the misspelled word.

Reviewer #5: Dear Editor,

The manuscript is clear and well written. The results and discussion are very interesting and contribute to the improve knowledge of a true and actual problem of dairy industry, which is to make longer the shelf life of high moisture mozzarella cheese. In This work the authors report the structural modification of the mozzarella cheese during freezing and thawing, a procedure used some dairy industry to increase the shelf life so that the mozzarella cheese can reach farther from production area. The authors cocncluded that a reduction of sensory and textural quality of the product occurs after thawing.

***Highlights (for review)**

- Water status in Mozzarella cheese changes during frozen and refrigerated storage
- Frozen storage induces microstructural damages and a reorganization of water phase
- Freezing-induced protein dehydration of cheese is observable by NMR relaxometry
- Textural and sensory properties of Mozzarella are partly affected by frozen storage

Water status and dynamics of high-moisture Mozzarella cheese as affected by frozen and refrigerated storage

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Abstract

High-moisture Mozzarella is one of the most exported cheeses worldwide, but ~~it is~~ affected by ~~a~~ short shelf-life~~torability~~. Freezing can help to ~~improve shelf life~~reduce waste ~~of the product~~, but its effect on quality needs to be considered. In this study, the physico-chemical changes of Mozzarella occurring during frozen storage and subsequent refrigerated storage (after thawing) were evaluated. Frozen cheeses stored at -18°C between 1 and 4 months showed microstructural damage and ~~s,~~ different physical, ~~water status,~~ textural, ~~and~~ sensory properties. With NMR relaxometry it was possible to observe freeze-related dehydration of caseins, by measuring the ~~as related to~~ changes in ~~the~~ water relaxation times ~~equilibrium~~ within the matrix. These modifications were confirmed by microstructural observations that showed the formation of larger serum channels in samples subjected to freezing, compared ~~to~~ with fresh ~~Mozzarella~~cheeses. Sensory evaluation showed skin peeling off in frozen samples. By observing the changes at various length scales ~~This approach helps~~it was therefore possible to identify ~~understand~~ the critical points affecting HM Mozzarella cheese quality ~~changes~~ during frozen storage.

Keywords: Frozen storage; Mozzarella cheese; Expressible serum; NMR relaxometry; Water holding capacity; Covering liquid

1. Introduction

Mozzarella is one of the most widespread cheese varieties worldwide. High moisture (HM) Mozzarella is a type of cheese ~~that is~~ characterized by a fresh, milky flavor and a soft consistency. Italian-style Mozzarella has a shelf life ranging from one to thirty days, and it is stored into a covering liquid. This kind of cheese shows specific properties and one of the most particular one is the release of droplets of serum of "milky" appearance when the cheese is cut (Mucchetti, Pugliese, & Paciulli, 2017). The covering liquid has a variable composition (i.e. water, lactic or citric acid, NaCl, $CaCl_2$ ~~etc~~) and it is a brine designed to keep the high moisture content in the cheese (usually more than 60%), maintain a very soft texture and prevent rind formation (Faccia, Gambacorta, Natrella, & Caponio, 2019; Mucchetti et al., 2017).

However, the use of covering liquid contributes to the high perishability of Italian Mozzarella, as it maintains high moisture content and water activity (a_w), with mass transfer between the cheese matrix and its serum phase (Faccia et al., 2019; Lucera et al., 2014).

Italian export of HM Mozzarella cheese has strongly increased over the last years. For example, of the 313,700 metric tons of cheese manufactured in Italy in 2017, 85,136 were exported (Assolatte, 2018), and more than 30% of the total export was shipped to extra-European countries (CLAL, 2020). Maritime~~Naval~~ transport would contribute to lower environmental impact, but it is not an option in the case of fresh Mozzarella (Dalla Riva et al., 2017); considering its short shelf life, fresh Mozzarella needs to be transported with rapid means ~~of transport~~ (e.g. air ~~transport~~ cargo).

In this view, freezing of HM Mozzarella cheese can improve storability of the product, and can contribute to a more robust supply chain, allowing to use slower, ~~but cheaper and~~ more sustainable means of transport (Alvarenga, Ferro, Almodôvar, Canada, & Sousa, 2013; Tejada et al., 2000). Freezing is currently applied to both low and high moisture Mozzarella cheese at industrial scale (Patent No. 2016/057931, 2017); however, most of the research has focused on low moisture commodity cheese (Bunker, 2016; Diefes, Rizvi, & Bartsch, 1993; Graiver, Zaritzky, & Califano, 2004). The effects of freezing and frozen storage on HM Mozzarella are quite different, due to the nature of the product, compared to LM Mozzarella, and need to be investigated to improve both process performances and product's quality.

Frozen storage has been shown to have a negative impact on LM Mozzarella cheese properties (Kuo, Gunasekaran, Johnson, & Chen, 2001). The formation of larger ice crystals during storage as a consequence of crystals growth and recrystallization can promote microstructural changes and the disruption of the casein matrix (Graiver et al., 2004; Kuo & Gunasekaran, 2009; Smith, Carr, Golding, & Reid, 2018); also the sensory attributes are affected (Park, Gerard, & Drake, 2006). A few studies have been ~~carried out~~reported on HM Mozzarella cheese subjected to freezing. Alinovi & Mucchetti~~,~~ (2020b) studied the effect of the presence or absence of covering liquid during freezing and the effect of different freezing and thawing rates. Conte et al.~~,~~ (2017) evaluated the effects of freezing rate and frozen storage on HM Mozzarella and highlighted a decrease of pores volume and sensory quality. However, there is still a gap of knowledge on the effect of freezing on HM Mozzarella cheese.

In cheese matrices, it is known that the changes in water dynamics and cheese microstructure promoted by freezing and thawing influence cheese quality characteristics (Kuo, Anderson, & Gunasekaran, 2003; Kuo & Gunasekaran, 2003). In particular, freezing and frozen storage promote protein dehydration and consequently textural and rheological changes in LM Mozzarella cheese (Diefes et al., 1993). During freezing, cooling is faster on the surface than in the center of the cheese; the concentration gradient then promotes water diffusion in the opposite direction from freezing (Smith et al., 2018). Moisture, solutes and temperature gradients affect the physical changes occurring to the product and their kinetics: accordingly, protein dehydration is a consequence of water migration phenomena that take place at a microscale level (Bunker, 2016; Reid & Yan, 2004).

In this context, due to the high moisture content and complex microstructure of Italian HM Mozzarella, the impact of freezing on the water dynamics will be critical and need to be ~~accurately~~ investigated. To study water dynamics and freezing-induced modifications in frozen-thawed foods, NMR spectroscopy is the method of choice (Gudjónsdóttir, Romotowska, Karlsdóttir, & Arason, 2019; Islam, Zhang, Fang, & Sun, 2015; Mortensen, Andersen, Engelsen, & Bertram, 2006; Sánchez-Valencia, Sánchez-Alonso, Martinez, & Careche, 2015; Xu, Zhang, Bhandari, Cheng, & Sun, 2015; Zhang, Haili, Chen, Xia, & Kong, 2018). According to the model of diffusive and chemical exchange (Belton & Hills, 1987; Hills, Wright, & Belton, 1989a, 1989b), the transverse relaxation curve in heterogeneous systems is usually multi-exponential.

NMR has been applied to study water dynamics in Buffalo and LM Mozzarella cheese (Gianferri, D'Aiuto, Curini, Delfini, & Brosio, 2007; Gianferri, Maioli, Delfini, & Brosio, 2007; Kuo et al., 2003, 2001). Kuo et al., (2003) demonstrated that the self-diffusion coefficient of water increased after frozen storage of LM Mozzarella cheese, for 4 weeks. It was shown that the water was less entrapped in the matrix, and this was attributed to the water release from the dehydration of casein matrix. ^{1}H T_2 relaxation times were shifted to lower values with frozen storage and the component distribution was narrower than in the fresh samples, pointing to the exchange of water molecules between phases with different mobilities (Kuo et al., 2003).

Gianferri, Maioli, et al., (2007) performed ^{1}H T_2 experiments on Buffalo Mozzarella cheese and observed a multi-exponential behavior because of the presence of different components, water and lipids, as well as of different structural elements in the sample. Different physical forms of water were described within the matrix: i) expressible water, corresponding to H_2O molecules located in the serum channels and pockets, whose motion is unaffected by the presence of the casein system; ii) entrapped water, corresponding to H_2O molecules inside the meshes of the casein network; iii) junction water, corresponding to H_2O molecules trapped within casein junction zones (Gianferri, Maioli, et al., 2007; McMahon, Fife, & Oberg, 1999). NMR is therefore a powerful technique to determine physical changes of Mozzarella cheese after freezing.

The objective of this work was to evaluate the effects of prolonged frozen storage and subsequent refrigerated storage after thawing on HM Mozzarella cheese characteristics, with a focus on the water status as measured at molecular level by ^{1}H NMR relaxometry. This methodology may be able to aid in understanding the molecular details of the freezing process in HM Mozzarella, and to highlight critical factors that can promote quality changes of the cheese during freezing and storage.

2. Material and methods

2.1 Experimental design

The experimental treatments were set up with a complete block design. A schematic representation of the experimental design can be observed in **Figure 1**. Three batches of HM Mozzarella were used in this experiment; each batch of cheese was assumed as the blocking factor of the design. For each batch, forty-five cheeses were frozen. To study the effect of the frozen storage on Mozzarella cheese properties, three

independent groups of fifteen cheeses for each batch were kept in frozen storage for 1, 3, and 4 months and then thawed.

Moreover, to study the influence of refrigerated storage, for each frozen storage group, three independent subgroups of frozen-thawed cheeses (n=5) were analyzed during the subsequent refrigerated storage, at 1, 3 and 8 days after thawing. For every batch, measurements were also performed on ~~the~~ a fresh, non-frozen cheese control group (n=15, coded as 0 month of frozen storage), considering the same days of refrigerated storage of the thawed cheeses.

2.2 Freezing conditions and experiments

Batches of fresh, HM Mozzarella cheese of 100 g were manufactured by Alival (Nuova Castelli S.p.a. RE, Italy). Cheeses used for the study were produced in different days within a period of two months. Cheeses were manufactured using standardized cow's milk (3.30 g/100 g protein, 3.50 g/100 g fat) that was pasteurized at 74 °C for 25 s. 1.2 g/100 g of citric acid and microbial rennet were added to coagulate milk. After cheese curd stretching, cheeses were moulded as 100-g individual spheroidal shapes; as each cheese was characterized by a non-regular spheroidal shape (Alinovi & Mucchetti, 2020a), it was characterized by a variable diameter of 4-6 cm. After moulding, cheeses were~~and~~ cooled by immersion into flowing tap water. Cheese composition was 18.0 g of protein, 17.0 g of fat, 1.0 g of lactose and 0.4 g of NaCl. Manufactured cheeses were kept at 4 ± 1°C into polyethylene bags containing 100 g of covering liquid (0.4 % w/w NaCl) for 6 days before being frozen.

Samples were separated from the covering liquid and then were frozen using an air blast freezer (MF 25.1, Irinox, TV, Italy) until the core of the cheese reached a temperature of -20°C (process time: 67 ± 3 min). Process was controlled by imposing an air temperature of -25°C and a velocity of 1.3 ± 0.2 m/s. Frozen cheeses were immediately vacuum packaged into polyethylene bags and stored at -18°C.

After reaching the predefined storage times (1, 3, 4 months), the independent groups of ~~samples~~ cheeses were thawed by applying an air temperature of +4°C, and a velocity of 1.3 ± 0.2 m/s. Applied freezing and thawing conditions were chosen in base of previous results, which showed that ~~as~~ these~~ey~~ conditions ~~did not~~

~~show a~~had no significant impact on cheese quality characteristics, when compared to faster freezing rates (Alinovi & Mucchetti, 2020b). After thawing, cheeses were immersed into 100 g of freshly prepared covering liquid (0.4 % w/w NaCl) and were stored in refrigerated conditions (4 ± 1°C). Before analyses, samples were taken out of the refrigerator and were equilibrated in a climate chamber (ICH 256L, Memmert, Schwabach, Germany) at 25.0 ± 0.1°C for 1 h.

2.3 Physical and chemical analyses

Changes in weight caused by the processes were measured by a laboratory scale (BCE 5200, Orma, Milan, Italy) with an accuracy of ± 0.1 g. Cheeses were weighted before freezing (fresh cheese), immediately after freezing, after thawing, and after overnight storage in fresh covering liquid (1-day post thawing). Weight variations were expressed as percentage changes of the original weight. Moisture Content (MC) of the cheese was measured in triplicate according to AOAC (1990).

Expressible serum of Mozzarella cheese (ES) was measured in triplicate by centrifuging 30 g of sample in 50 mL falcon tubes at 12,500 g per 75 min using a centrifuge (mod. 5810 R, Eppendorf, Hamburg, Germany) according to Guo & Kindstedt (1995). After centrifugation, the fat layer (supernatant) was removed, the serum was transferred into another tube and the quantity was measured by weight using an analytical scale (mod. AR 2140, Ohaus Corporation, New Jersey, USA). ES was expressed as percentage of the weighted serum (ES_{app}) related to the MC of the cheese, according to equation 1:

$$ES\% = \frac{ES_{app}}{MC} \times 100 \quad (1)$$

After centrifugation and fat layer removal, pH and electrical conductivity of ES were respectively measured with a Portamess pH-meter and a conductometer mod. 913 (Knick Elektronische, Berlin, Germany), respectively equipped with a Double Pore F electrode (Hamilton Company, Reno, Nevada, USA) and a TetraCon 325 probe (WTW Xylem Analytics, Weilheim, Germany) having a cell constant (K) of 0.475 cm^{-1}.

2.4 Low-field NMR analyses

NMR analyses were performed using a low resolution ^{1}H NMR spectrometer (the Minispec, Bruker, Massachusetts, USA) with a frequency of 20 MHz and a magnetic field strength of 0.47 T. Temperature during the analyses was set at 25.0 ± 0.1 °C using an external thermostatic bath (Julabo F30, Julabo Labortechnik GmbH, Seelbach, Germany).

Mozzarella cheese samples were cut from the central part of the cheese and transferred into an NMR tube (outer diameter of 10 mm) that was filled up to 10 mm height; to avoid moisture loss during the analysis, the tube was sealed by using a thermoplastic laboratory film.

^{1}H T_1 spin-lattice ~~longitudinal~~ relaxation time~~s were~~ was determined by the inversion-recovery pulse method. The sequence was performed using a recycle delay (RD) of 3 s (> 5 ^{1}H T_1); the first and last pulse spacing (t) between the 180° and 90° pulses were 0.1 ms and 2,500 ms, respectively, and 20 data points were acquired. Eight scans were performed for each measurement.

^{1}H T_2 ~~transverse~~ spin–spin relaxation curves were measured with a Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence (Meiboom & Gill, 1958), by performing sixteen scans for each replication, with a RD of 3 s (> 5 ^{1}H T_1), an interpulse spacing (τ) of 40 μs and 16,000 data points.

^{1}H T_1 and ^{1}H T_2 curves were analyzed as quasi-continuous distributions of relaxation times using an UPENWin software (Alma Mater Studiorum, Bologna, Italy, Borgia, Brown, & Fantazzini, 1998, 2000). Default values for all UPEN settings parameters were used with the exception of the LoXtrap parameter that was set to 1 to avoid extrapolation of relaxation times shorter than the first experimental point. T_1 and T_2 relaxation curves were also fitted with multiexponential models using Sigmaplot, v.10 (Systat Software Inc., USA) according to previous works (Diantom et al., 2019; Gianferri, Maioli, et al., 2007), as follows:

~~$A_1(t) = L_1 + \sum_i A_{1(i)}(1 - e^{-\tau/T_{1(i)}})$~~ ~~(2)~~

$$M(t) = \sum_i M_{\infty(i)}(1 - 2e^{-\tau/T_{1(i)}}) \quad (2)$$

$$A_2(t) = L_2 + \sum_i A_{2(i)} \cdot e^{-\tau/T_{2(i)}} \quad (3)$$

Where ~~A_1~~$M(t)$ $A_2(t)$ are the T_1, T_2 amplitude exponential functions, $T_{1(i)}$, $T_{2(i)}$ are the spin-lattice and spin–spin relaxation times, respectively, of component *i*, , $M_{\infty(i)}$~~$A_{1(i)}$~~, $A_{2(i)}$ are the spin-lattice maximum magnetization value at equilibrium and spin–spin signal ~~intensities~~intensity, respectively, of component *i*, and the constant ~~L_1~~, L_2 ~~are~~ is the intersect~~s~~ of the polynomial function~~s~~ and represent the instrumental noise of the measurements. Each Mozzarella cheese sample was analyzed in quadruplicate for both ^{1}H T_1 and T_2 analyses.

2.5 Light microscopy

Microstructural characterization of Mozzarella cheese ~~samples~~ over the frozen storage time considered was performed by a slightly modified procedure proposed by Noronha et al. (2008).

Samples were analyzed using an Olympus bx51 light microscope (Olympus Corp., Tokyo, Japan) equipped with a 10x objective lens. Disks of cheese (30 mm diameter, 4 mm thickness) taken from the central part of a cheese sample were cut at -40°C in cryo-sections (6 μm thickness) using a cryo-microtome (MTC Benchtop, SLEE Mainz, Mainz, Germany). Sampled sections were dried and subsequently fixed using a 2.5% aqueous glutaraldehyde solution for 3 min. Fixed specimens were stained using aqueous fast green (0.5 g / 100 mL) for 3 min to stain proteins and subsequently rinsed using Milli-Q water. Samples were then immersed into aqueous triethyl phosphate (40 g / 100 mL) (TEP) for 30 s, and then stained with Sudan III (1 g / 100 mL TEP) for 25 min to color fat. Finally, stained samples were rinsed with Milli-Q water and examined using the microscope. Observations were made in quadruplicate one day after thawing, at every frozen storage time considered (0, corresponding to the control, non-frozen cheese, 1, 3, 4 months).

2.6 Texture Profile Analysis

Cheese texture was measured at room temperature using a TA.XT2plus texture analyzer (Stable Micro Systems, Godalming, UK). Measurement replicates (n=5) were cut in small cubes (15×15×15 mm) using a knife.

Texture Profile Analysis (TPA) test was performed using a stainless-steel cylindrical probe with a diameter of 30 mm. Samples were compressed to 60% strain by applying a crosshead speed of 1.5 mm/s. Textural parameters considered were hardness (N), cohesiveness, springiness and gumminess (N).

2.7 Descriptive sensory analysis

Quantitative descriptive analysis was performed by five panelists (three males, two females) that were trained according to Alinovi & Mucchetti (2020b), and that had previous experience with descriptive sensory analysis. Evaluated sensory descriptors were chosen from a list of Mozzarella cheese descriptors (Pagliarini, Monteleone, & Wakeling, 1997). Selected sensory descriptors were sensory juiciness, acidity, saltiness, translucency, paste smoothness, surface smoothness. The intensity of every descriptor was evaluated between 1 (absence of the attribute) and 9 (extreme intensity of the attribute). Cheeses were ~~portioned~~ prepared in portions of 10 mm cubes for taste and aroma evaluation, while a half portion of the cheeses was used for visual evaluation; samples were equilibrated at 25°C prior to each assessment.

2.8 Statistical analysis

The main effect of frozen storage (Ft_i, i=0, 1, 3, 4 months, with 0 months corresponding to the fresh, control cheese), and refrigerated storage (Rt_k, k=1, 3, 8 days) and the significance of their interactions were evaluated by creating split-plot ANOVA models for all the ~~analysed~~analyzed parameters using PRC GLM of SAS (SAS Inst. Inc., NC, USA) according to Alinovi, Rinaldi, & Mucchetti (2018). ~~Batch~~ The batch of cheese (B_j, j=1, 2, 3) was used as the blocking factor of the models (equation 4):

$$Y_{ijkl} = \mu + Ft_i + B_j + \delta_{ij} + Rt_k + (Ft \times Rt)_{ik} + \gamma_{ijk} \quad (4)$$

Where δ_{ij}, and γ_{ijk} are the main plot and subplot error terms, respectively; Y_{ijkl} is the selected response variable. Post hoc tests were performed by Tukey's honest significant differences test ($\alpha = 0.05$) when significant main effects and interactions were found.

Sample classification was also carried out using a multivariate approach with principal component analysis (PCA). Prior to analysis, variables were normalized.

Pearson's correlation coefficients (r) were also calculated to find relations among evaluated variables. Bivariate correlation and PCA analysis were performed using SPSS v.25 (IBM, Armonk, USA).

3. Results and discussion

3.1 Physical and chemical characteristics

Frozen Mozzarella cheeses showed a weight decrease after ~~subsequently~~ freezing (residual weight of 98.4 ± 0.2%, relatively to the original fresh cheese weight), that was further slightly enhanced by the thawing process after frozen storage (97.5 ± 0.7%). ~~However, after~~After submersing the cheese ~~being immersed~~ in fresh covering liquid, immediately after thawing, frozen-thawed cheeses regained weight, as a consequence of water absorption from the covering liquid. Therefore, the ~~; as a consequence,~~ weight difference between fresh cheeses ~~weighted~~ before and after ~~-~~freezing and thawing, after ~~and frozen-stored cheeses subjected to the~~ immersion in covering liquid ~~after thawing,~~ was negligible (residual weight of 100.0 ± 1.0%). This was fully in line with previous reports~~, according with the results of a previous work~~ (Alinovi & Mucchetti, 2020b). In addition, a~~Also, a~~s expected, there was no significant variation in weight ~~observed~~ during ~~the~~ frozen storage time (P=0.690). Weight change after the overnight period was also not influenced by the length of refrigerated storage (P=0.718). The moisture content (MC) of control fresh and frozen-thawed cheeses after different frozen storage times was not significantly different (P>0.05) (result not shown).

The fraction of unbound water contained in Mozzarella cheese, estimated by measuring the expressible serum (ES), as reported in **Table 1**, showed a significant increase with frozen storage (**Table S1, supplementary material**). Already after the first month of frozen storage, the amount of ES was significantly higher (+3.5%) than that measured in the fresh cheese (**Table 1**). An increase of ES after frozen storage is a clear evidence for water rearrangements; in the past, this has been attributed to casein dehydration as a consequence of the formation, rearrangement and growth of ice crystals (Kuo & Gunasekaran, 2009), and conformational changes in protein structure (Fontecha, Bellanato, & Juarez, 1993). This can have a substantial impact at a macroscopic level. During frozen storage, water released from the

casein matrix migrates in serum channels: the result is a decrease of bound water and a consequent increase of unbound water. After thawing, the dense protein network is no longer able to re-adsorb bulk water. This will result in a harder cheese matrix, as there is a diminished lubrication effect on the gel network structure (Bertola, Califano, Bevilacqua, & Zaritzky, 1996).

The mean electrical conductivity of the ES was ~~also~~ measured, as also shown in **Table 1**. There were no differences in the values for both *Ft* and *Rt* variables. On the other hand, there was a slight decrease of pH of ES with frozen storage with values at the limit of significance (P=0.05) (**Table 1**).

The change in pH is attributed ~~can be explained by~~ to the ~~possible~~ precipitation of insoluble calcium phosphate because of frozen storage and the ~~; this would~~ decrease in ~~the~~ buffering capacity of the medium. In previous studies, the decrease of pH was also found to be related to caseins precipitation ~~phenomena~~ caused by changes in ionic equilibria (Kljajevic et al., 2016; Tribst, Ribeiro, Leite Junior, de Oliveira, & Cristianini, 2018). Statistical analysis demonstrated that the pH values are negatively correlated to ES (r=-0.502).

3.2 ^{1}H T_1, T_2 NMR results

Representative ^{1}H T_1 relaxation curves of Mozzarella cheese samples are reported in **Figure ~~1~~2**. The longitudinal relaxation curves were characterized by the presence of two resolved ^{1}H components. The fastest relaxation component, indicated as A in **Figure ~~1~~2**, was characterized by a relaxation time ranging between 0.9 and 2.6 ms, and it represented between 22.4 and 3.6 % of total protons, depending on the storage history of Mozzarella samples. On the other hand, the slowest ~~relaxing~~ relaxation component, (indicated as B) was the most prominent, and showed a relaxation time between 176 and 381 ms, with a relative percentage ranging between 77.6 and 96.4%.

The two components A and B can be attributed to protons of water molecules that are bound (A) or free diffusing (B), but also to solid (A) or liquid (B) fat phase (Mariette, 2009). The bound water can be associated to macromolecules such as caseins or to the lipid interface. It is possible to hypothesize that microstructural or chemical changes occurring to product undergoing frozen and refrigerated storage will

affect ^{1}H relaxation and chemical exchange processes (Gianferri, Maioli, et al., 2007) and NMR is a good means to test changes in *in situ* during freezing or after storage.

Statistical analysis of longitudinal relaxation times and relative abundances of components A and B showed a significant difference as a function of both frozen (*Ft*) and refrigerated time (*Rt*) (**Table S2, supplementary material**). **Figure 1** illustrates the NMR relaxation curves for frozen and refrigerated times~~different frozen (F_t) and refrigerated times (R_t)~~. **Figure ~~2~~ 3** illustrates the relative abundance of the component at fast relaxation times (A) as a function of freezing times. At longer frozen storage times~~r F_t~~ there was a significant decrease of the water molecules with fast relaxation times (**Figure ~~2~~3**), while the relative percentage of the slower component (B) increased. The increase of relative abundance of the slow ~~relaxing~~ relaxation component B was related to the migration of water molecules that are strongly associated to the casein structure and/or to chemical groups containing reactive protons (Gianferri, Maioli, et al., 2007), to the interstitial voids that are surrounded by the protein domain (serum channels), increasing the ~~free~~ percentage of free diffusing water. ~~This migration of water is related to freeze-induced protein dehydration and related to the increase of ES during the frozen storage; in particular the increase of component B was significantly related to the increase of ES (r=0.380).~~

There were significant changes in the T_1 relaxation times, as a function of refrigerated storage (*Rt*), and in particular on the mobility of the molecules distributed in peak B (**Figure ~~1~~2**). The component with faster relaxation time shifted to longer values after storage.~~refrigerated storage.~~ For example, while T_{1B} at the first day of refrigerated storage of fresh, non-frozen Mozzarella was 214 ± 12 ms, after 8 days shifted to 282 ± 85 ms. After 1, 3, 4 months of frozen storage, a similar shift of T_{1B} during refrigerated storage was observable: T_{1B} shifted from 246 ± 38 ms, 227 ± 32 ms, 241 ± 36 ms at the first day of refrigerated storage for 1, 3, 4 months of frozen storage ~~*Ft*~~, respectively, to 281 ± 39 ms, 305 ± 32 ms, 285 ± 48 ms. A similar shift to longer relaxation components for ^{1}H T_1 was also reported by a previous study on low moisture (LM) Mozzarella cheese (Kuo et al., 2001): in this case it was hypothesized that this increase in relaxation time in the early stages of storage is related to proteins structural rearrangements, because of the dynamic, non-quiescent state of caseins.

In our study, changes shown in T_1 relaxation times and relative abundance of proton populations can not be attributed to protein hydration, as observed during the refrigerated storage of LM Mozzarella cheeses (Guo & Kindstedt, 1995; Kuo & Gunasekaran, 2009; Kuo et al., 2001); there was a different behavior, confirmed by both NMR and ES measurements, caused by freezing. In particular, the migration of water from population A to population B can be related to freeze-induced protein dehydration and related to the increase of ES during the frozen storage; the increase of component B was significantly related to the increase of ES (r=0.380). As freezing can promote modification of the tertiary and quaternary structure of proteins (Xiong, 1997), it may lead to irreversible dehydration of the protein matrix. Furthermore proteolysis, which is present during frozen storage (Meza, Verdini, & Rubiolo, 2011), can also have an impact.

Figure ~~3~~ 4 reports the ^{1}H T_2 relaxation curves for various HM Mozzarella samples as a function of freezing and subsequent refrigerated storage. ^{1}H T_2 signal can give different information than ^{1}H T_1, because it includes additional relaxation mechanisms (i.e., exchange of nuclei between different environments) (Kuo et al., 2001; Lucas, Wagener, Barey, & Mariette, 2005). The ^{1}H T_2 behavior showed the presence of four ^{1}H components, as previously shown in the case of Buffalo Mozzarella cheese (Gianferri, D'Aiuto, et al., 2007).

In ^{1}H T_2, there was only one completely resolved component (A) that relaxed in the range between 0.1 and 4.2 ms and it peaked~~),~~ at very short relaxation times (~ 0.1-0.2 ms~~~ 1-2 ms~~) (**Figure ~~3~~4**). This component corresponds to the ^{1}H of water strongly bound to the casein structure as solvation water ~~**(Figure 3)**~~ (Gianferri, Maioli, et al., 2007). The results indicated that there were clear differences in the mobility of the ^{1}H with freezing time. Component A showed a significant increase of its mobility with both frozen (*Ft*) and ~~refridgerated~~refrigerated (*Rt*) storage times and (**Figure 5a, b**), while its relative abundance did not change (P>0.05, **Table S2, supplementary material**).

The three long relaxation times components (components B, C, D having relaxation times of ~20 ms, ~60-80 ms, ~300-400 ms, respectively) represented the major components in the ^{1}H T_2 curves and they were partially overlapped (**Figure ~~3~~4**).

~~The results indicated that there were clear differences in the mobility of the ^{1}H with freezing time. Component A showed a significant increase of its mobility with both *Ft* and *Rt* (**Figure 4a, b**), while its relative abundance did not change (P>0.05, **Table S2, supplementary material**).~~

As shown in **Figure ~~3~~ 4** during *Ft*, ^{1}H T_2 components exhibited a broadening of their peaks and the overlapping zones of components B, C, D increased, indicating a more inhomogeneous protons exchange and molecular structure (Littardi et al., 2019).

Components B and C, were assigned to protons of water trapped in the protein meshes and to the lipids contained into the fat globules ~~in~~ of Mozzarella di Bufala Campana cheese, respectively (Gianferri, D'Aiuto, et al., 2007). However, the characteristics of this cheese are different due to the higher moisture content and the fat/casein ratio of buffalo Mozzarella cheese compared to ~~are quite different from~~ HM Mozzarella~~, for the higher moisture content and fat/caseins ratio of the first one~~ (Mucchetti et al., 2017). In our study, it was not possible to make a clear distinction between the two components, as they were largely overlapped (**Figure ~~3~~4**). It was hypothesized that both components were constituted by ^{1}H related to the fat phase and to the trapped water molecules. At longer frozen and refrigerated times (*Ft* and *Rt*), component B decreased its relative abundance, while component C increased ~~it~~ (**Figure 4~~c~~5c, d**). This may be ~~possibly because of the~~caused by the water/fat phase less bound ~~and entrapped~~ by the casein matrix at longer storage times, as already explained in the case of T_1 curves. Similarly to T_{1B}, component T_{2C} was found to be significantly correlated with ES (r=0.473). Finally, the relaxation time of component C showed a slight increase during *Rt* ($P<0.05$), changing from 69 ± 8 ms at 1st day to 77 ± 5 ms at 8th day of refrigeration~~*Rt*~~.

The slowest relaxation population D, that can be related to a part of the physically trapped water according to Gianferri, Maioli, et al. (2007) did not show a significant change in both relaxation time and relative abundance, as a consequence of frozen and refrigerated storage ($P>0.05$).

3.3 Microstructural changes

Microstructural changes of HM Mozzarella cheeses during frozen storage can be observed in **Figure ~~5~~6**. Changes in the microstructure of the cheese were observed already after 1 month of frozen storage at -18°C, ~~if~~ when compared with ~~the~~ control, non-frozen cheese. Changes were still evident during the subsequent frozen storage times (3 and 4 months). However, there was only a slight microstructural variation at increasing storage times (1, 3, 4 months). As is it possible to observe, frozen storage promoted the formation of relatively bigger fat globule~~s~~ clusters and larger serum channels surrounded by the protein matrix. These

results are in accordance ~~to the~~with the observed increase of ES (**Table 1**) and the ~~, to the~~ modifications of the molecular dynamics observed by ^{1}H T_1 and ^{1}H T_2 NMR relaxometry. Furthermore, the results support prior ~~, and to~~ microstructural observations ~~performed in other studies~~ (Graiver et al., 2004; Kuo & Gunasekaran, 2009), that ~~observed~~ reported an increase in pores size and rupture of the protein network in frozen stored LM Mozzarella cheese using scanning electron microscopy. These observations also agree with the hypothesis that freezing and frozen storage can lead to local dehydration phenomena of the protein matrix; while water is released by the protein matrix, and ice crystals are formed between casein fibers, an increase of serum channels and pockets can be observed after thawing. Volume increase of the water fraction that becomes ice, can also lead to favor the contact between fat globules, that are more prone to form clusters and agglomerates (Diefes et al., 1993).

3.4 Textural properties

Textural analyses showed a significant variation of hardness and gumminess as a function of frozen storage time~~*Ft*~~ ($P<0.05$, **Table S1 supplementary material**). Hardness and gumminess significantly increased both after the first and third month of frozen storage; on the contrary, from the third to the fourth month, both parameters decreased and showed similar values to the fresh control (**Table 2**). The increase of hardness and gumminess until the third month of frozen storage was attributed to the dehydration of the protein network. As previously discussed, frozen storage promoted the rearrangement~~s~~ and the consequent formation of a more compact protein matrix, with aggregates of casein that interact each other (i.e. formation of disulphide bridges) and are intercalated by serum channels and fat clusters of bigger dimensions that have a lower lubricant effect (Alvarenga, Canada, & Sousa, 2011; Diefes et al., 1993). On the contrary, a softening of the cheese texture from the third month of frozen storage can be caused by protein hydrolysis~~, as shown by other authors~~ (Meza et al., 2011).

Also, frozen storage showed a significant effect on cohesiveness: fresh, non-frozen cheeses were found to be different from the frozen cheeses, already from the first month of frozen storage. The results shown in Table 2 were in full agreement with previous work (Alinovi & Mucchetti, 2020b). In this case, control cheese was more cohesive than frozen-stored cheeses, due to changes in microstructure and moisture organization ~~due~~

~~to~~caused by protein dehydration. The freeze-induced reorganization and the damage of the casein matrix caused the formation of a more plasticized structure. Statistical analysis showed that the cohesiveness values were weakly negatively correlated to protein dehydration (e.g. with NMR ^{1}H T_1 component B, r=-0.415).

3.5 Sensory properties

From a sensory point of view, cheeses showed differences in appearance, with changes in surface smoothness, while the other parameters (sensory juiciness, acidity, saltiness, translucency, paste smoothness) were not affected by both *Ft* and *Rt* (P>0.05, **Table S1, supplementary material**).

A significant increase of the amount of imperfections on the surface of the cheese was observed during frozen storage. **Figure ~~6~~ 7** illustrates the difference in appearance between fresh cheese and cheese frozen for 4-months. However, sensory tests showed an obvious change in smoothness already after one month of frozen storage (**Figure ~~7~~8**). This peel off of the cheese's surface is usually reported after prolonged refrigerated storage of HM Mozzarella cheese into covering liquid (Laurienzo et al., 2008), enhanced by the caseinolytic activity of some non-lactic acid bacteria directly on the cheese surface (Baruzzi, Lagonigro, Quintieri, Morea, & Caputo, 2012).

After freezing, the peel off can be attributed to the~~the abrupt~~ changes in ~~of~~ thermal and physical properties caused by ~~as a consequence of~~ temperature ~~changes~~ fluctuations and inhomogeneities during freezing and thawing, especially to the outer part of the cheese, ~~to the growth of ice crystals during frozen storage, and to temperature inhomogeneities in the outer part of the cheese during freezing and thawing, which are~~ due to the irregular geometry of Mozzarella cheese (Alinovi & Mucchetti, 2020a). The peel off can also be caused by the growth of ice crystals during frozen storage. In particular, it can be hypothesized that changes in volume ~~changes~~, can promote the partial separation of connected fibrous layers of the cheese matrix, with the result that a small part of Mozzarella cheese skin lost in the covering liquid. Moreover, also ~~colloidal calcium phosphate depletion caused by the increasing ionic strength~~changes in ionic equilibria during freezing (Kljajevic et al., 2016)~~,~~ can be responsible for surface disruption phenomena (Faccia et al., 2019).

PO~~n the contrary, p~~arameters such as juiciness and translucency that are sensorial parameters that can be sensorially related to the water status and serum organization in Mozzarella cheese (Pagliarini et al., 1997), were not significantly affected by refrigerated or frozen storage times~~both *Rt* and *Ft* factors~~. These results would suggest that the extent of water status modifications measured using NMR and ES methods, despite being significant, may be not sensorially perceived.

3.6 Samples classification according to PCA

Principal component analysis (PCA) was performed to have an overview and a classification of the cheeses as a function of frozen storage (**Figure ~~8~~9, ~~9~~10**). The multivariate analysis generated three PCs that explained 58.4% of variance of the dataset. The low variance explained can be due to variability encountered in relation with the batch of cheese, as already observed in the case of univariate analyses.

A classification pattern between fresh and frozen stored-cheeses was observed, and it was mainly caused by PC2 (**Figure ~~8a~~9a**); while the separation was low when comparing fresh and 1-month frozen stored cheeses, it was strong when comparing fresh cheeses and 3, 4-months frozen stored cheeses. In **Figure ~~8a~~9a**, samples having increased frozen storage times, were characterized by lowered scores on PC 2; on the contrary, fresh cheeses were always characterized by positive scores on PC 2. Moreover, as reported in **Figure ~~8b~~9b,** *Rt* did not cluster the cheeses in relation of the measured parameters.

By observing the loading plot (**Figure ~~9~~10**), PC2 was mainly represented by positive loadings of ~~percentages of components~~ T_{2B} and T_{1A} component relative abundances, textural cohesiveness, pH of the serum phase. On the contrary, relative ~~percentage~~ abundance of T_1, T_2 more mobile proton populations (T_{1A}, T_{2C}), T_{1A}, T_{2A}, T_{2C} relaxation times, expressible serum, surface smoothness showed negative loading of PC2.

Interestingly, the pH of serum phase was found to be well correlated to the relative percentage of component~~s~~ T_{1A} (r=0.781) and negatively correlated to textural hardness (r=-0.501). Moreover, a correlation between sensory juiciness and the relative percentage of the more mobile T_{2D} component was also present (r=0.502); this water population can be assumed as the fraction of the expressible serum that is weakly, physically held by the cheese matrix, that is in exchange with the water of the covering liquid of HM

Mozzarella cheese and that is sensorially perceived during tasting. Juiciness was also found to be weakly correlated to electrical conductivity of ES (r=0.433).

By comparing the loading and score plots, fresh cheeses were mainly differentiated from the frozen thawed cheeses for their different water status and mobility, lower ES, higher cohesiveness.

4. Conclusions

Frozen HM Mozzarella cheeses stored at -18°C for a period ranging between 1 and 4 months showed ~~some~~ differences compared to the ~~than~~ fresh samples. ~~Mozzarella cheeses; f~~Frozen cheeses were affected by freezing induced microstructural damage~~s~~, different water status and water holding capacity, and changes in ~~different~~ textural and ~~,~~ sensory properties, compared to ~~than~~ fresh Mozzarella cheeses.

With low field NMR relaxometry, it was possible to measure dehydration ~~phenomena~~ of caseins induced by prolonged frozen storage. The work identified some critical mechanisms linked to the ~~se are critical points that must be considered when storing HM Mozzarella cheese in freezing conditions, as they can considerably~~ reduction of ~~e the~~ refrigerated shelf life after thawing, and the sensory and textural quality of the product.

These results can be useful to understand how to control and limit ~~the critical points that affects HM Mozzarella cheese frozen storage and to find possible ways that can limit~~ the degree of modifications of the matrix.

Conflicts of Interest

The authors declare no potential conflicts of interest.

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Figure Captions

Figure 1. Schematic representation of the experimental design applied in this study.

Figure ~~1~~2. ^{1}H T_1 NMR relaxation curves of HM Mozzarella cheese samples at different frozen storage (*Ft*) (0, corresponding to fresh non frozen cheeses, 1, 3, 4 months) and subsequent refrigerated storage (*Rt*) times (1, 3, 8 days). ^{1}H T_1 components are indicated as component A and B in the graph.

Figure ~~2~~3. Variation of relative percentage of ^{1}H T_1 component A of HM Mozzarella cheeses stored for different frozen storage times (*Ft*); data are reported as means of all refrigerated storage times. Different letters indicate significantly different means ($P<0.05$).

Figure ~~3~~4. ^{1}H T_2 NMR relaxation curves of HM Mozzarella cheese samples at different frozen storage (*Ft*) and refrigerated storage (*Rt*) times. Reported curves were: fresh, non-frozen cheese samples stored for 1 and 8 days of refrigerated storage (0.1, 0.8, respectively); 1, 3 4 months frozen stored cheese samples subsequently stored for 8 days of refrigerated storage (1.8, 3.8, 4.8, respectively). Different ^{1}H T_2 proton populations are indicated as components A, B, C, D in the curves.

Figure ~~4~~5. Variation of ^{1}H T_2 NMR variables of HM Mozzarella as a function of different frozen and refrigerated storage times (*Ft, Rt*): ^{1}H T_2 relaxation time of component A (a, b), and relative percentage of components B and C (c, d). Different letters indicate significantly different means ($P<0.05$). The graphs were built by averaging the data as a function of all refrigerated storage times (panel a, c) and of all frozen storage times (panel b, d).

Figure ~~5~~6. High moisture Mozzarella cheese microstructure observed at different frozen storage periods, 1 day after thawing: (**a)** 0 month (fresh, non-frozen control cheese), (b) 1 month, (c) 3 months, (d) 4 months. PM: protein matrix; FG: fat globule; FC: fat cluster; CA: serum channel.

Figure ~~6~~7. Comparison between the external surface of fresh (a), and 4-months frozen stored (b) HM Mozzarella cheeses.

Figure ~~7~~8. Surface smoothness score as a function of frozen storage time; data are reported as means of all refrigerated storage times. Surface smoothness was sensorially evaluated by performing a QDA test using a

trained panel group (n=5). Values are expressed as mean score points (minimum score 0, maximum score 9) evaluated by a trained panel group (n=5). Different letters indicate significantly different means (P<0.05).

Figure ~~8~~9. Principal component analysis (PCA) score plots (a, b). Principal components were calculated considering all the parameters evaluated in this study. Samples were labelled according to the different frozen storage period (A) (○ = 0 month, □ = 1 month, ◇= 3 months, ▲= 4 months) and refrigerated storage period (B) (○ = 1 day, □ = 3 days, ▲= 8 days).

Figure ~~9~~10. Principal component analysis (PCA) loading plot. Principal components were calculated considering all the parameters evaluated in this study.

Table 1. Physical, chemical parameters as a function of the different frozen storage times (~~0 month, corresponding to the~~ fresh cheese, 1, 3, 4 months of frozen storage), reported as means of all refrigerated storage times of HM Mozzarella cheeses. Measured parameters were expressible serum (ES), pH and electrical conductivity (COND) of ES.

Frozen storage (months)	ES (%)	pH (-)	COND (mS cm^{-1})
0	$52.9^{c} \pm 2.3$	$5.92^{a} \pm 0.04$	$9.0^{a} \pm 2.5$
1	$56.4^{b} \pm 1.9$	$5.82^{b} \pm 0.12$	$8.8^{a} \pm 1.9$
3	$57.0^{ab} \pm 2.4$	$5.73^{c} \pm 0.06$	$9.6^{a} \pm 1.4$
4	$58.1^{a} \pm 2.2$	$5.73^{c} \pm 0.06$	$9.3^{a} \pm 2.1$

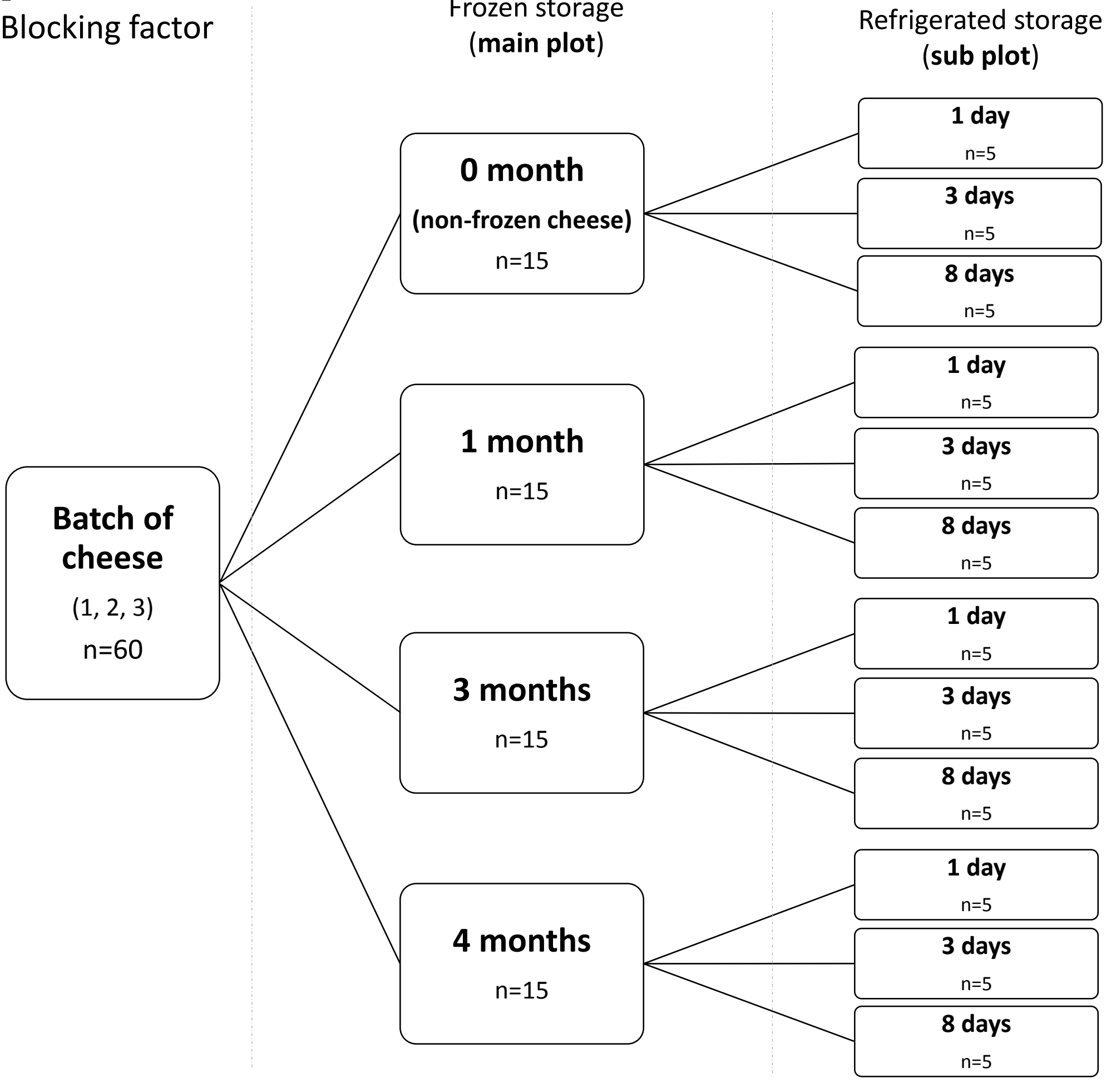
$^{a-c}$ Mean values within a column with different superscript letters are significantly different ($P<0.05$).

Table 2. Textural parameters as a function of the different frozen storage times (~~0 month, corresponding to the~~ fresh cheese, 1, 3, 4 months of frozen storage), reported as means of all refrigerated storage times of HM Mozzarella cheeses. Measured parameters were hardness, cohesiveness, gumminess, springiness.

Frozen storage (months)	Hardness (N)	Gumminess (N)	Cohesiveness (-)	Springiness (-)
0	$19.1^{c} \pm 7.2$	$11.8^{c} \pm 4.5$	$0.62^{a} \pm 0.03$	$0.73^{a} \pm 0.04$
1	$23.9^{b} \pm 7.7$	$14.1^{b} \pm 4.8$	$0.59^{b} \pm 0.02$	$0.71^{a} \pm 0.04$
3	$26.3^{a} \pm 5.6$	$15.3^{a} \pm 3.7$	$0.58^{b} \pm 0.02$	$0.72^{a} \pm 0.04$
4	$19.7^{c} \pm 5.2$	$11.4^{c} \pm 3.1$	$0.58^{b} \pm 0.01$	$0.75^{a} \pm 0.06$

$^{a-c}$ Mean values within a column with different superscript letters are significantly different ($P<0.05$).

Figure 1_new


Blocking factor
Frozen storage
(main plot)
Refrigerated storage
(sub plot)
Batch of cheese
(1, 2, 3)
n=60
0 month
(non-frozen cheese)
n=15
1 month
n=15
3 months
n=15
4 months
n=15
1 day
n=5
3 days
n=5
8 days
n=5
1 day
n=5
3 days
n=5
8 days
n=5
1 day
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n=5
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n=5
1 day
n=5
3 days
n=5
8 days
n=5

Figure 2

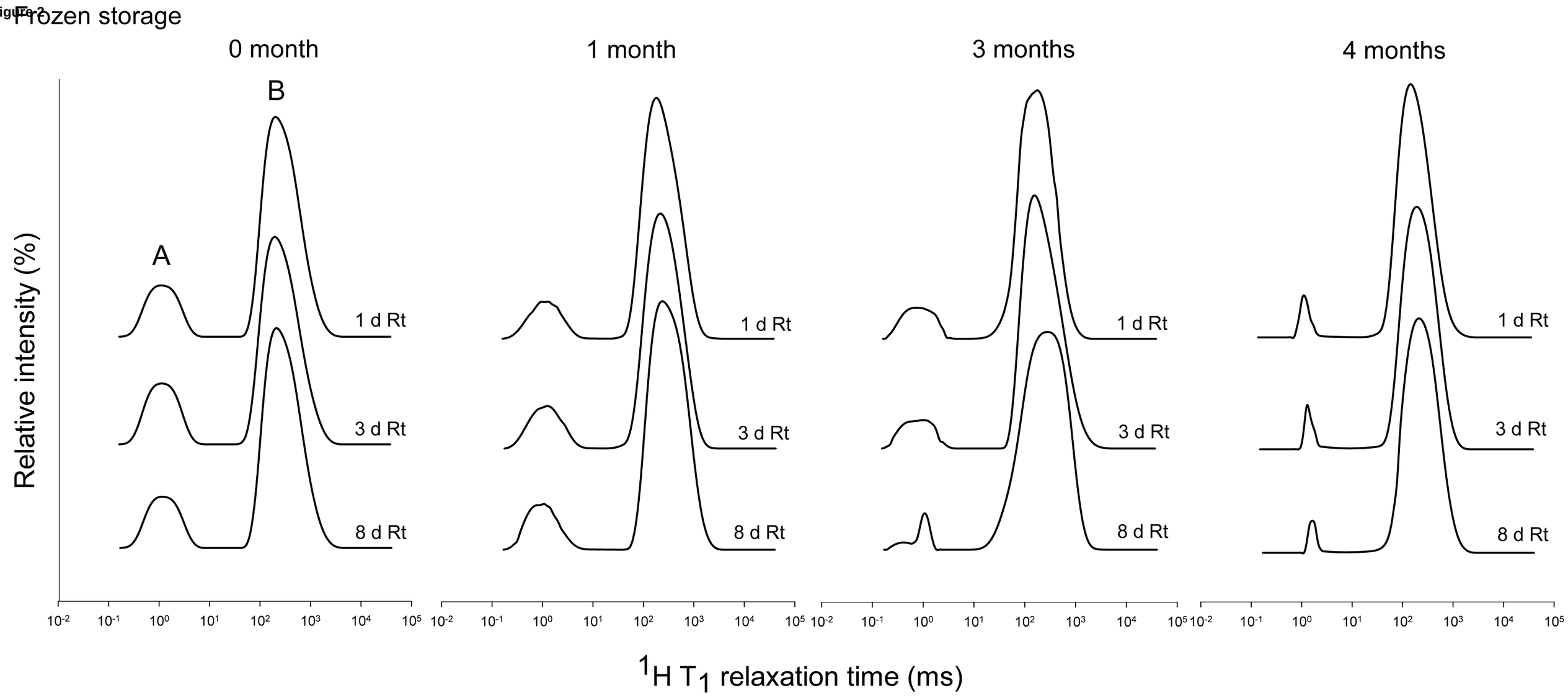

Frozen storage
0 month
1 month
3 months
4 months
A
B
1 d Rt
3 d Rt
8 d Rt
Relative intensity (%)
10-2 10-1 100 101 102 103 104 105
1H T1 relaxation time (ms)

Figure 3

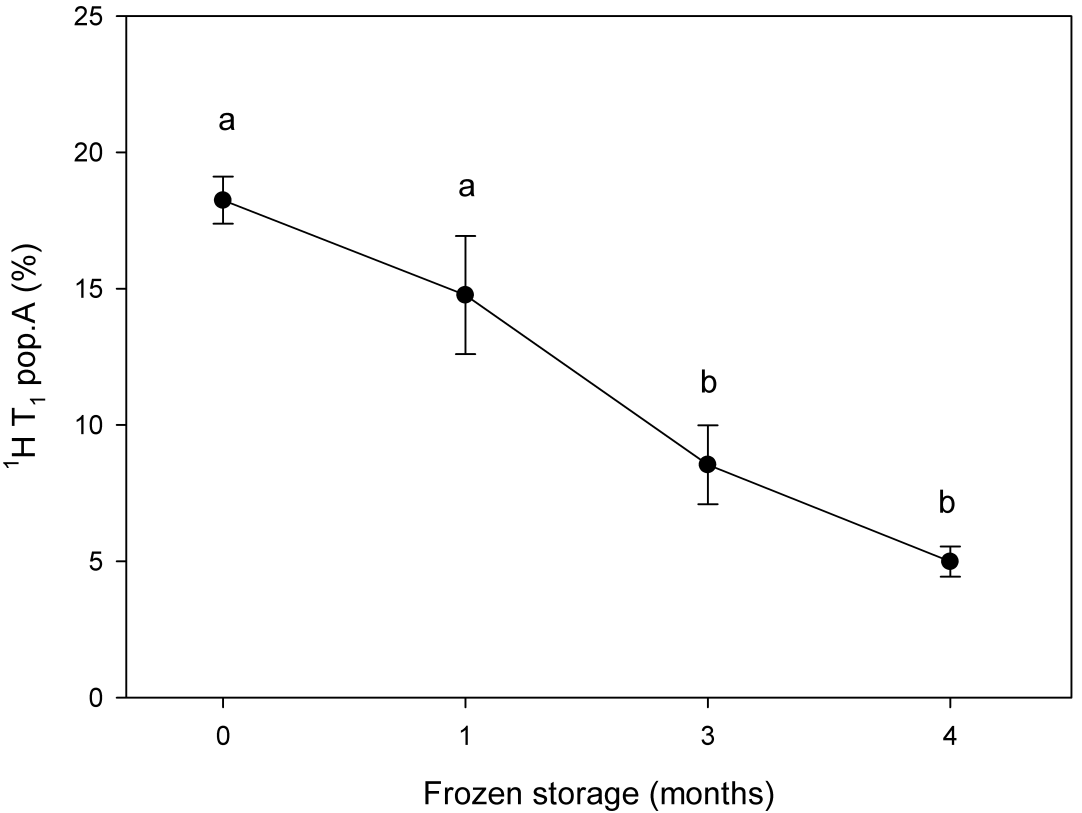


Figure 4

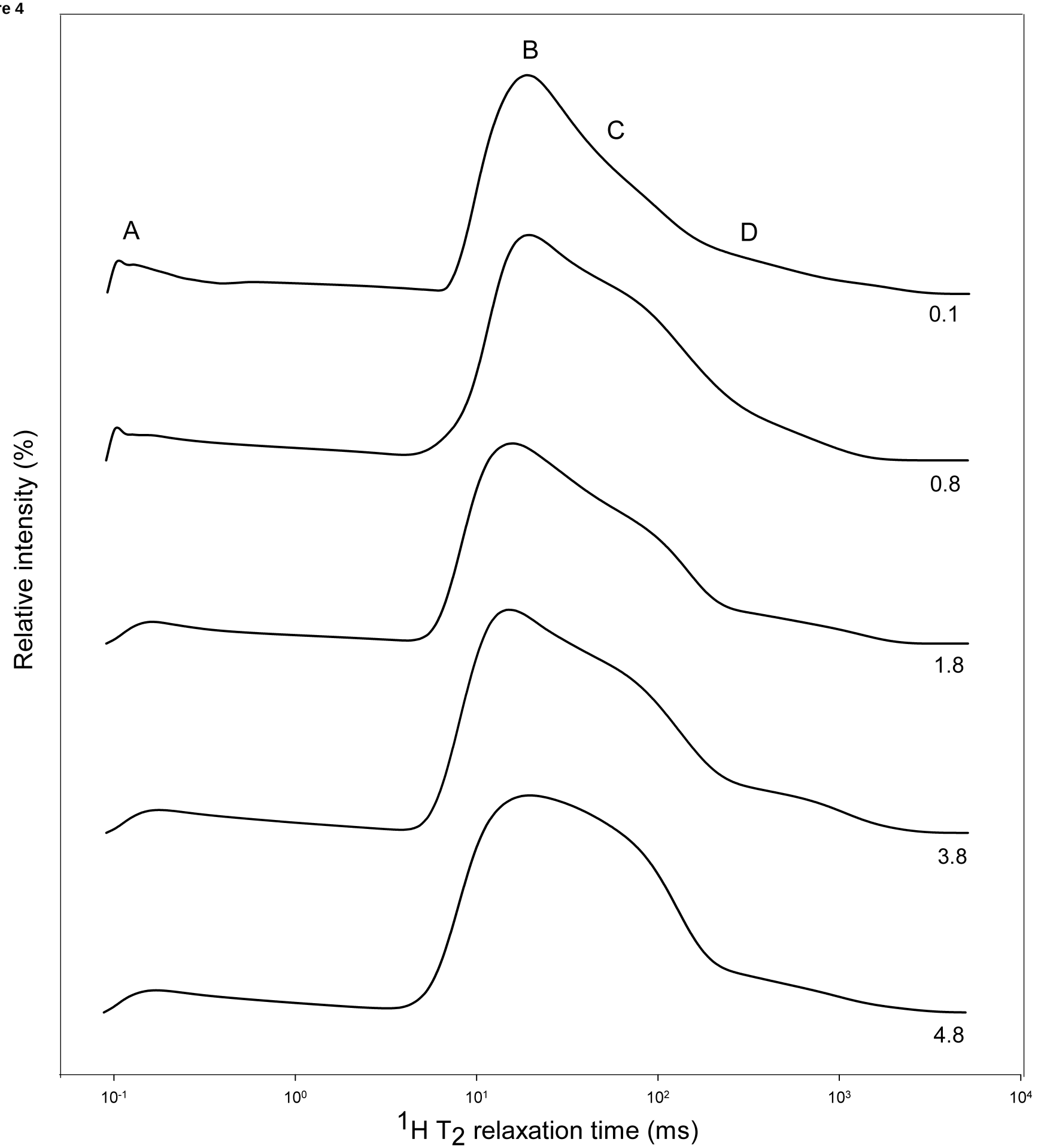
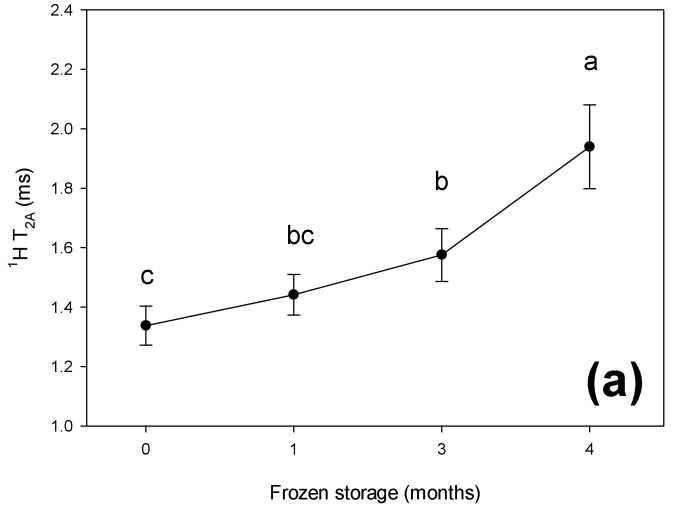
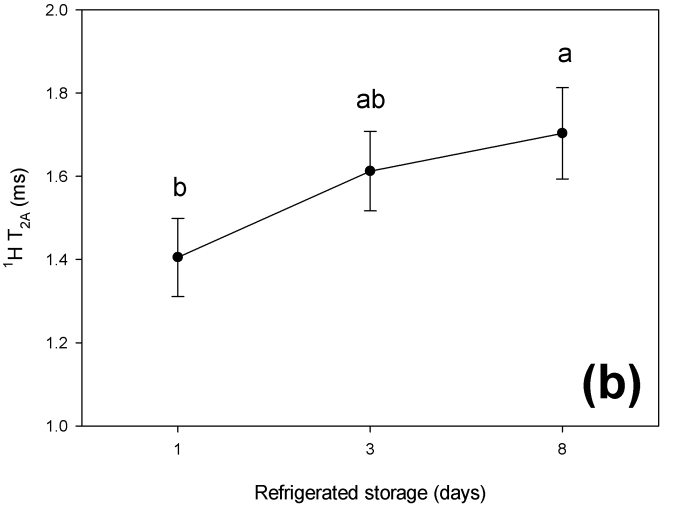
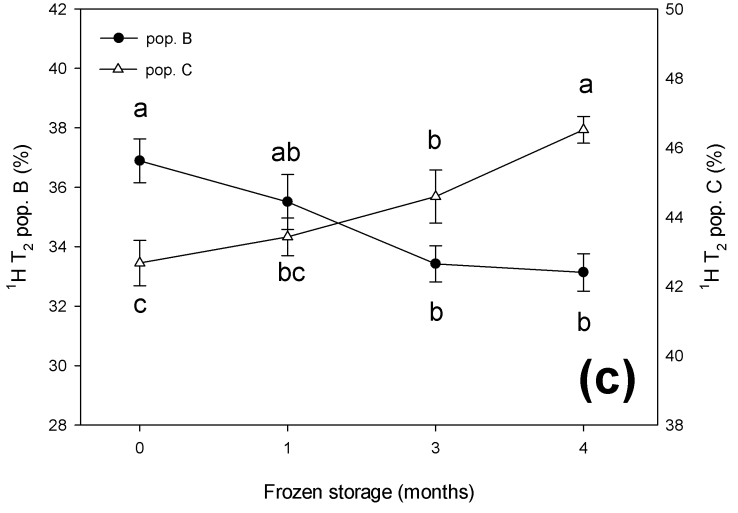

B
C
A
D
0.1
0.8
1.8
3.8
4.8
Relative intensity (%)
10-1
100
101
102
103
104
1H T2 relaxation time (ms)

Figure 5_revised







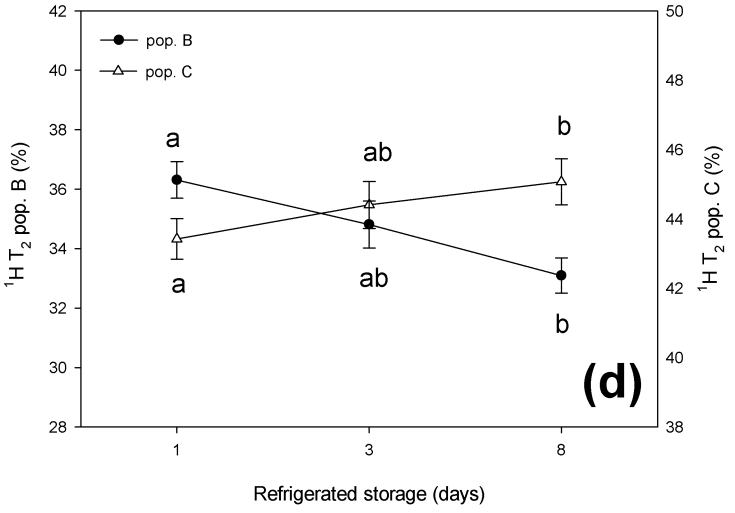


Figure 6

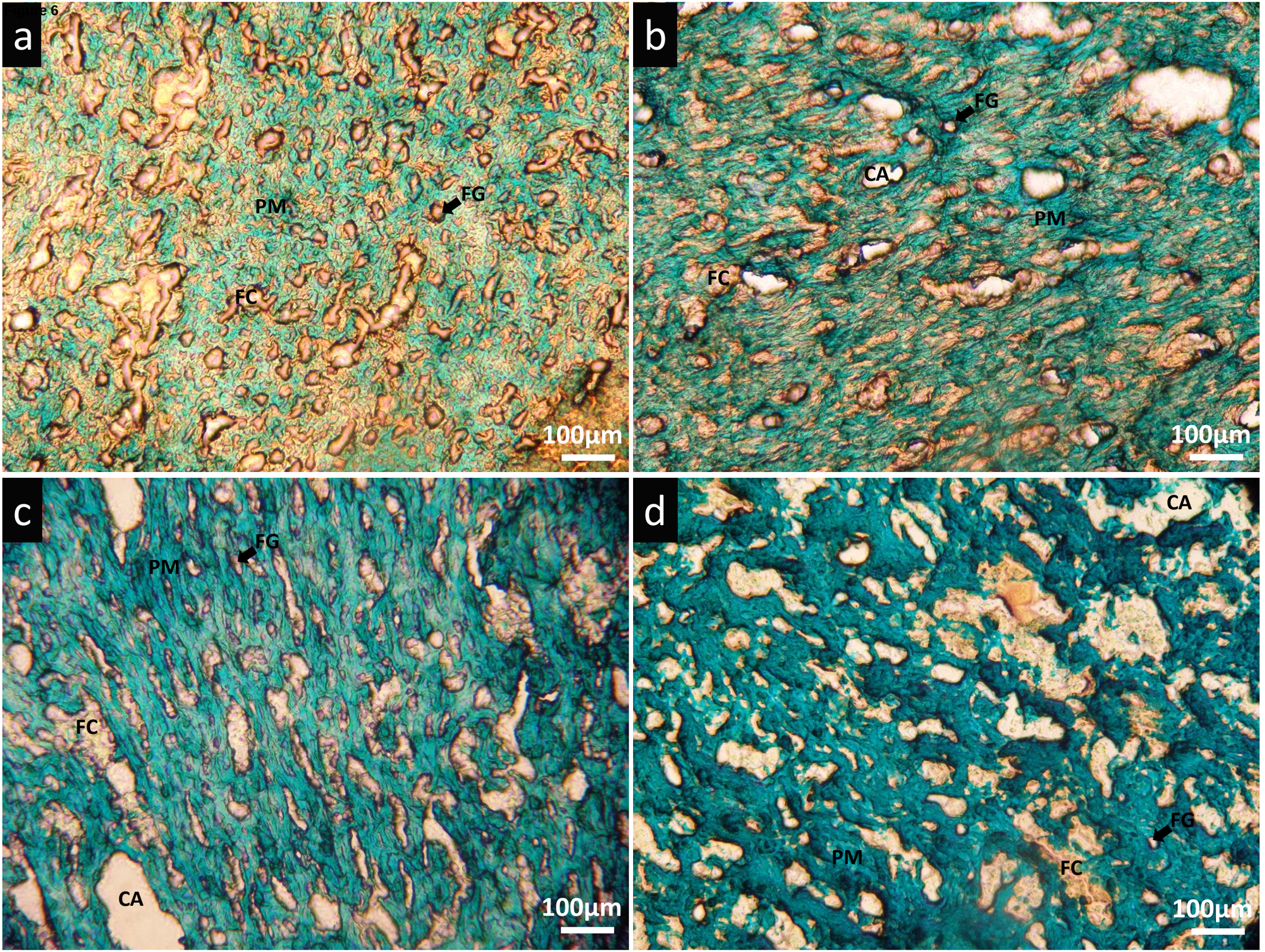

a
PM
FG
FC
100μm
b
FG
CA
PM
FC
100μm
c
FG
PM
FC
CA
100μm
d
CA
FG
PM
FC
100μm

Figure 7

Click here to download high resolution image

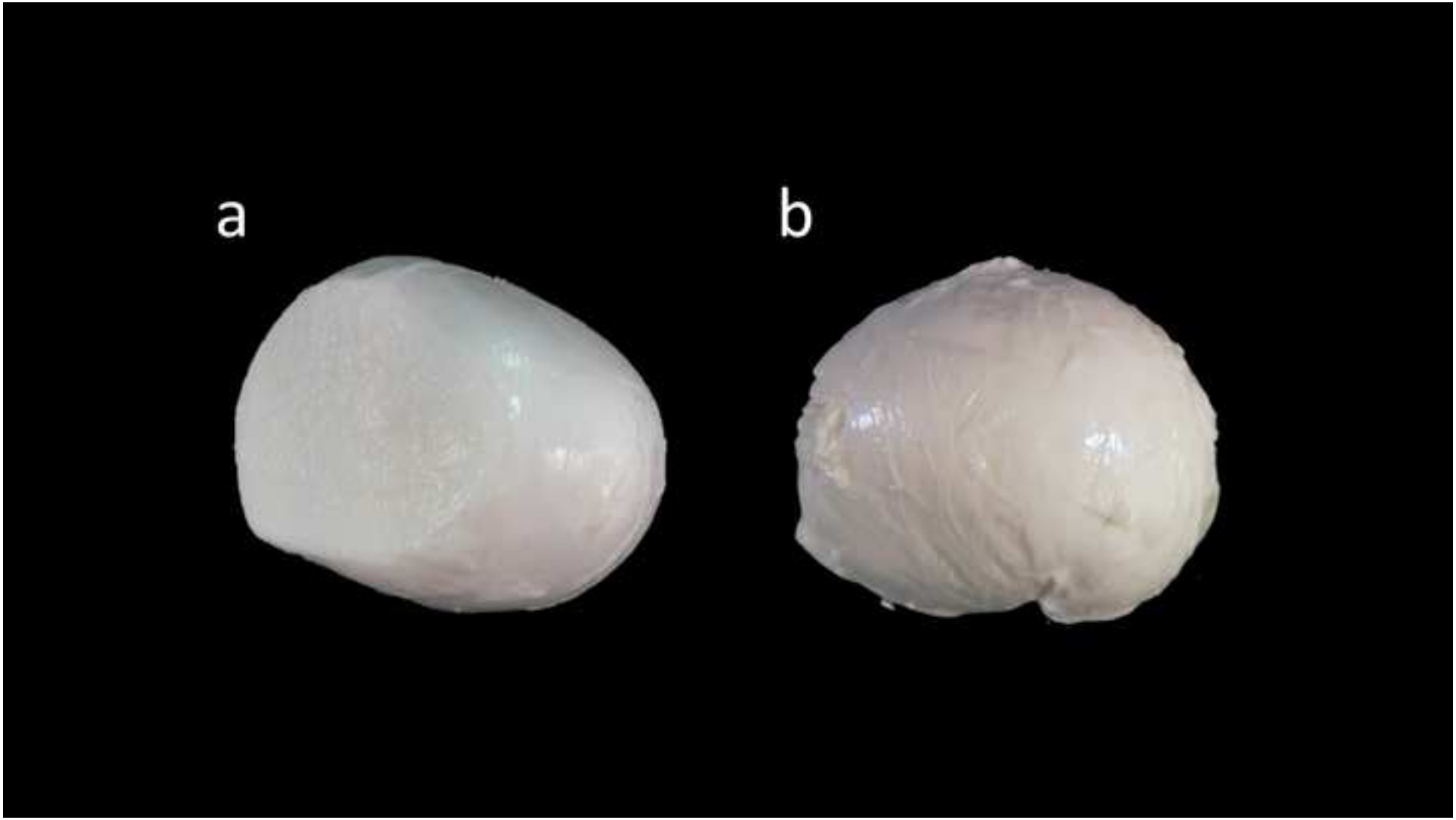


Figure 8

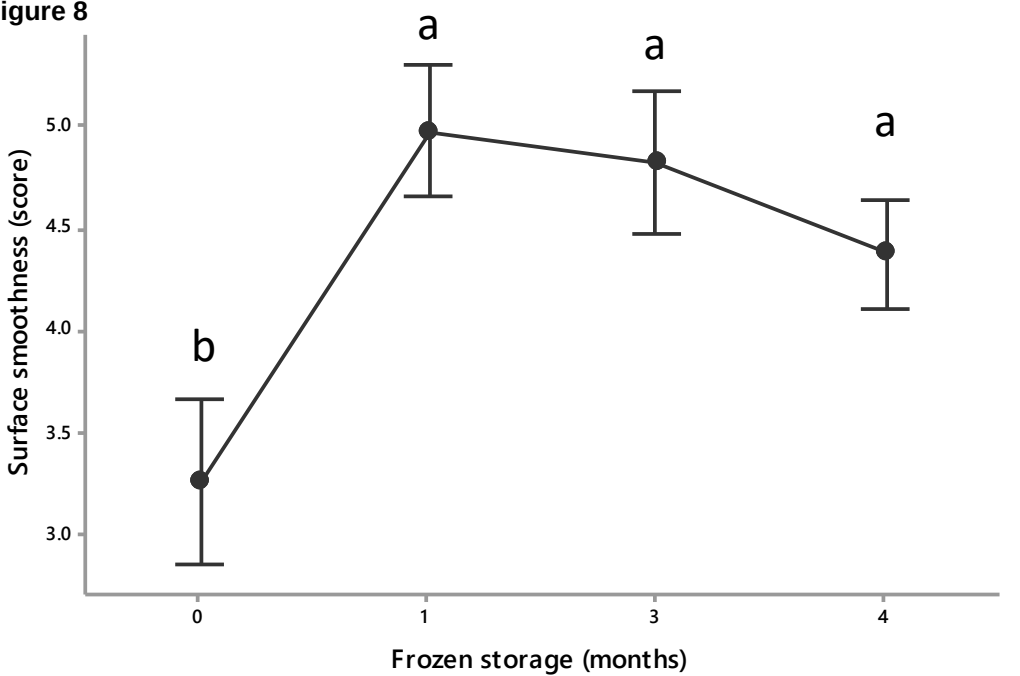
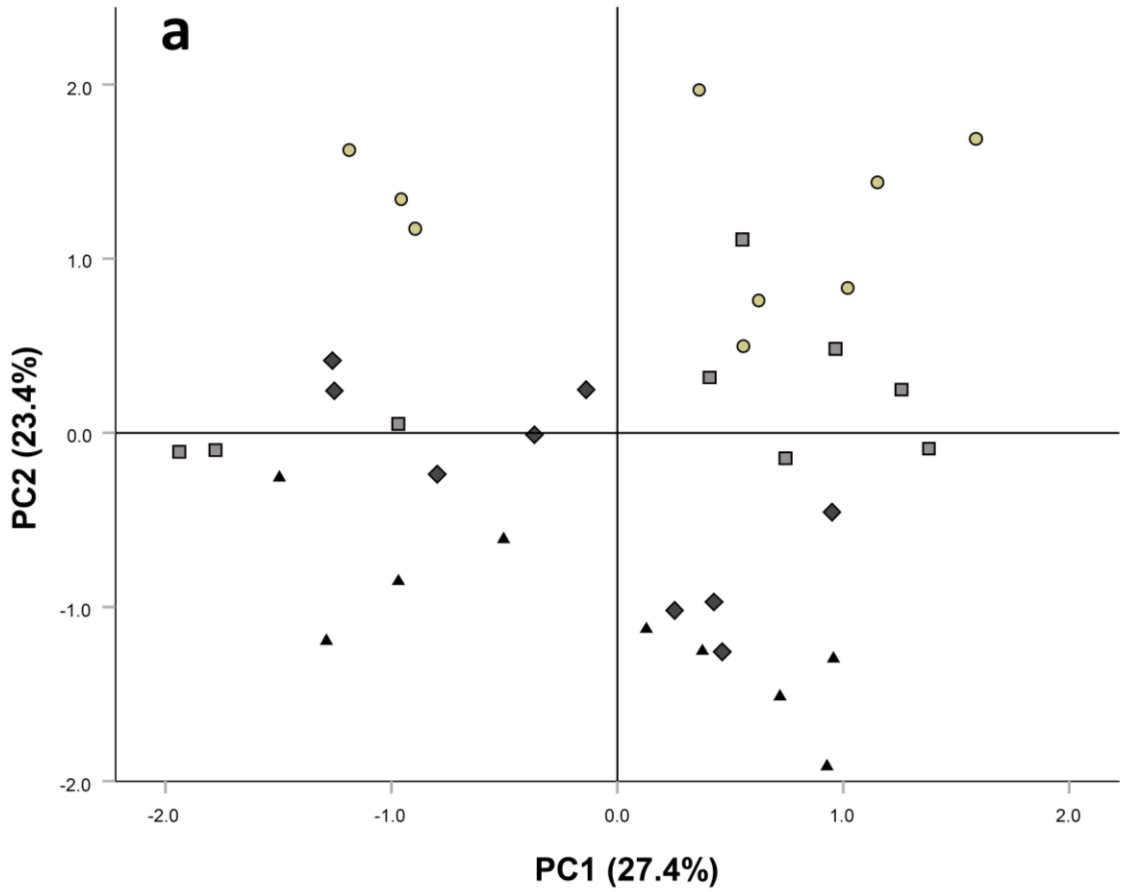

a
a
a
b
5.0
4.5
4.0
3.5
3.0
Surface smoothness (score)
0
1
3
4
Frozen storage (months)

Figure 9


a
2.0
1.0
0.0
-1.0
-2.0
PC2 (23.4%)
-2.0
-1.0
0.0
1.0
2.0
PC1 (27.4%)

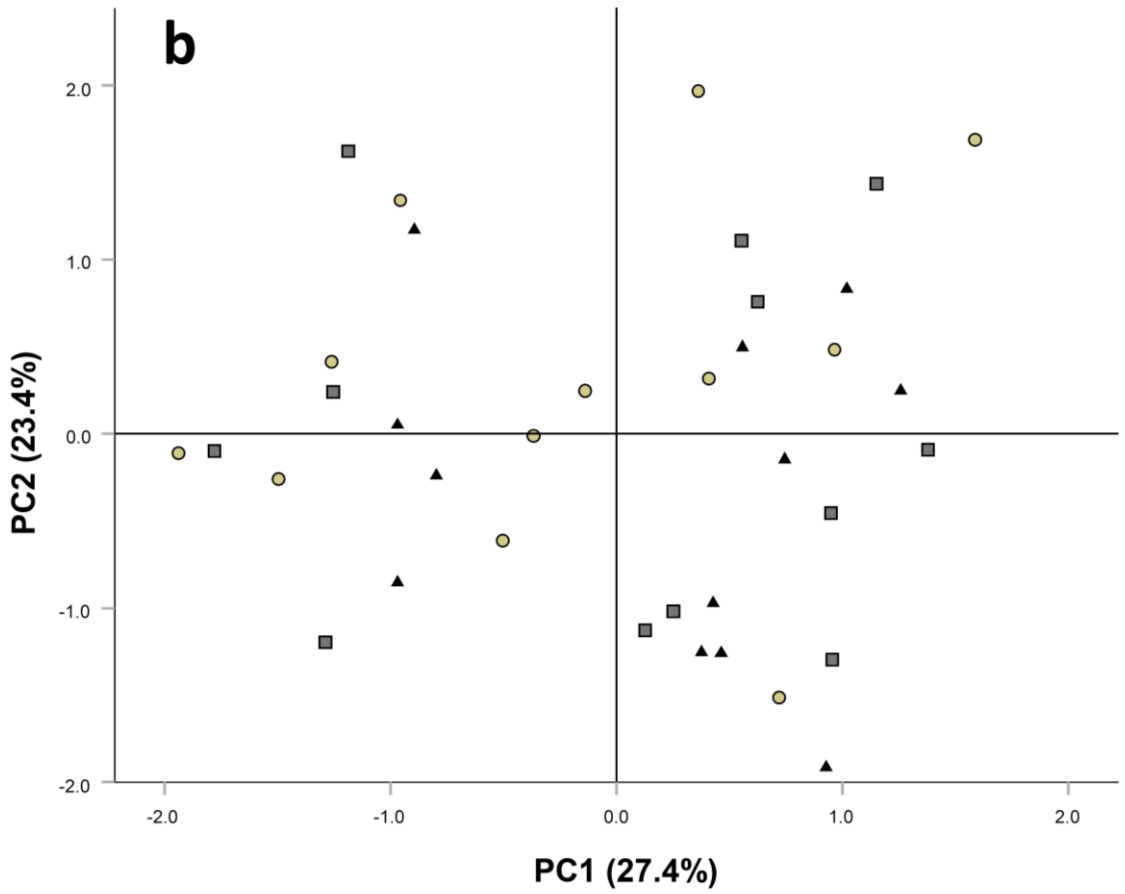
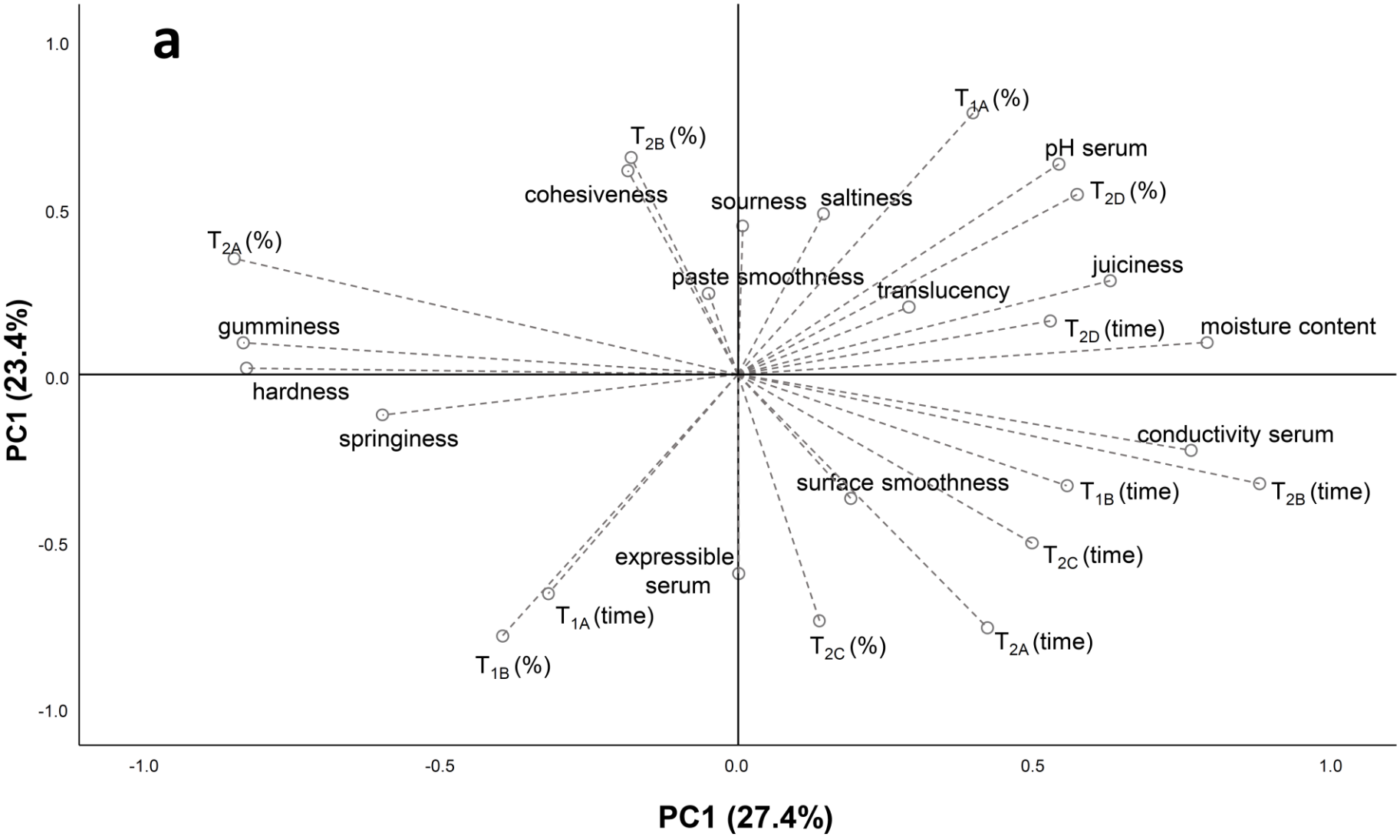

b
2.0
1.0
0.0
-1.0
-2.0
PC2 (23.4%)
-2.0
-1.0
0.0
1.0
2.0
PC1 (27.4%)

Figure 10


a
1.0
0.5
0.0
-0.5
-1.0
PC1 (23.4%)
-1.0
-0.5
0.0
0.5
1.0
PC1 (27.4%)
T1A (%)
T2B (%)
pH serum
T2D (%)
cohesiveness
sourness
saltiness
T2A (%)
paste smoothness
translucency
juiciness
gumminess
T2D (time)
moisture content
hardness
springiness
conductivity serum
surface smoothness
T1B (time)
T2B (time)
T2C (time)
expressible serum
T1A (time)
T2C (%)
T2A (time)
T1B (%)

Supplementary material for online publication only

Click here to download Supplementary material for online publication only: Supplementary_Data_05_03_20.pdf

***Graphical Abstract**

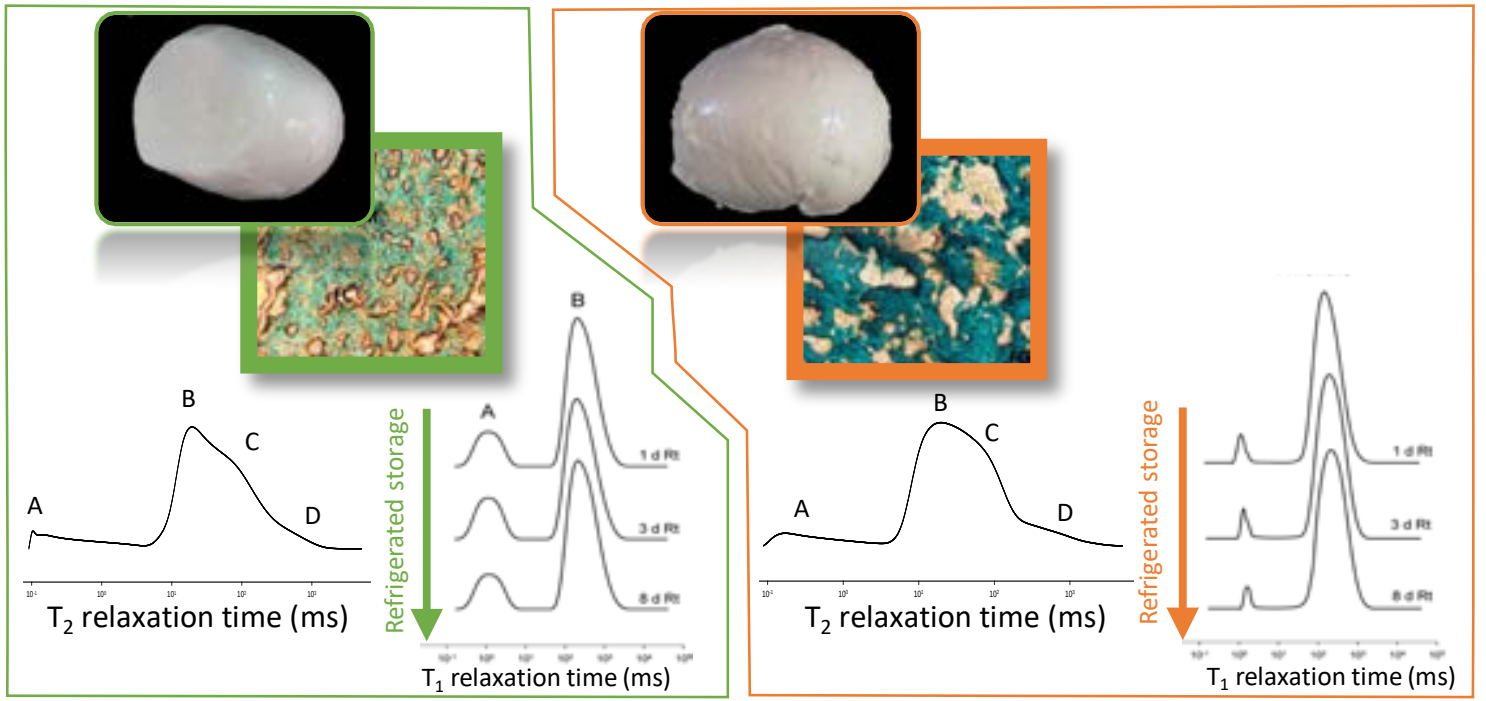
MOZZARELLA CHEESE

Frozen storage:
0, 1, 3, 4 months

-18°C

Refrigerated storage:
1, 3, 8 days

+4°C



Credit Author Statement

Marcello Alinovi: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Data Curation, Writing - Original Draft. Milena Corredig: Writing - Review & Editing, Visualization. Germano Mucchetti: Conceptualization, Visualization, Investigation, Resources, Writing - Review & Editing, Supervision, Project administration, Funding acquisition. Eleonora Carini: Methodology, Software, Validation, Resources, Writing - Review & Editing.

***Conflict of Interest form**

Declaration of interests

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

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