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Author/s:
Pax, AP;Ong, L;Pax, RA;Vongsvivut, J;Tobin, MJ;Kentish, SE;Gras, SL

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Industrial freezing and tempering for optimal functional properties in thawed Mozzarella cheese

Anita P. Pax[a,b], Lydia Ong[a,b], Randolph A. Pax[c], Jitraporn Vongsvivut[d], Mark J. Tobin[d], Sandra E. Kentish[a], Sally L. Gras*[a,b]

[a] The ARC Dairy Innovation Hub, The Department of Chemical Engineering, The University of Melbourne, Parkville, Victoria 3010, Australia

[b] The Bio21 Institute, The University of Melbourne, Parkville, Victoria 3010, Australia

[c] RAP Innovation and Development Pty Ltd, PO Box 559, Indooroopilly, Queensland 4068, Australia

[d] Infrared Microspectroscopy (IRM) Beamline, ANSTO – Australian Synchrotron, 800 Blackburn Road, Clayton, Victoria 3168, Australia

*Corresponding author: The Bio21 Molecular Science and Biotechnology Institute, The University of Melbourne, Parkville, Victoria 3010, Australia. Tel.: +61 3 8344 6281; Fax: +61 3 8344 4153. E-mail address: sgras@unimelb.edu.au

Abstract

Mozzarella cheese was industrially frozen (-18 °C), stored for up to six months, tempered at 4 °C for one or three weeks and the structure and functionality compared to cheese stored at 4 °C and cheese aged at 4 °C for four weeks prior to freezing. When combined with ageing or tempering, the slow industrial freezing minimised changes to the protein network as detected by confocal microscopy and arrested proteolysis. Cheese functionality improved with three weeks of tempering, with properties similar to cheese refrigerated for one month, potentially due to increased proteolysis and protein rehydration. Frozen storage induced β-sheet and β-turn structures, as detected by S-FTIR microspectroscopy, with longer tempering leading to structural recovery in the cheese. This study indicates the proteolysis and functionality of frozen cheese can be optimised with tempering time. It also provides new insights into heat transfer during the industrial freezing and tempering of cheese.

Keywords

Microstructure; dairy; meltability; protein structure; freezing; frozen; temperature profile; heat transfer;

1. Introduction

Freezing is widely used in the food industry to preserve product quality, extend shelf life and allow product distribution to more distant export markets (Kuo & Gunasekaran, 2009; Reid & Yan, 2004). Freezing may be achieved by air blast chilling, storage in natural convection freezers or by individual quick freezing (IQF), where individual product units or particles are frozen before or after packaging. Food may also be stored at low temperature in an unfrozen state, using a process called supercooling, where the product structure may be retained and energy saved as the latent heat of the product is not removed (Stonehouse & Evans, 2015). A drawback of supercooling, however, is that this process does not provide the same extension to shelf life as freezing, highlighting the importance of ongoing studies that can improve our understanding of the freezing of food products.

The freezing point for Mozzarella cheese ranges from -1.2 °C to -5 °C, depending on the conditions applied (Diefes, Rizvi, & Bartsch, 1993; Ribero, Rubiolo, & Zorrilla, 2007). This variability arises from the concentration of solutes in the aqueous phase, with salt being a key solute that depresses the freezing point (Ribero et al., 2007). Other factors, such as Mozzarella age, free moisture content and cheese structure may also affect the freezing temperature.

Structural damage can occur during freezing leading to a change in cheese properties (Fontecha et al., 1996; Verdini, Zorrilla, & Rubiolo, 2005). Such damage is likely via two mechanisms: firstly the phase change and thermal expansion from water to ice may physically alter the protein network; and secondly local dehydration may alter protein structure (Diefes et al., 1993; Fontecha, Peláez, Juárez, & Martín-Hernández, 1994; Kuo & Gunasekaran, 2009). Local dehydration of proteins may occur when water that is free or entrapped within the network freezes. Not all water freezes, however, with some vicinal and multilayer water molecules remaining unfrozen and associated with hydrophilic sites within the cheese matrix (Coultate, 2009). Changes to protein hydration may also alter protein secondary structure, which can be detected by spectroscopy (Pax et al., 2019).

Both the rate at which the cheese is cooled to the freezing point and the length of time for conversion to a solid phase likely influence the extent of structural damage (Oberg, Merrill, Brown, & Richardson, 1992). To date, most experimental studies have applied cooling rates that are much faster than those that occur in industrial situations. Prior studies report reductions in temperature from -1.1 °C to -6.7 °C occurring over a period of 0.2-10 hours compared to ~100 hours at commercial scale (Bertola, Califano, Bevilacqua, & Zaritzky, 1996; Diefes et al., 1993; Fontecha et al., 1996; Kuo & Gunasekaran, 2003; To et al., 2020). This situation is in part due to samples being smaller than those industrially frozen, i.e., 1.5-2.5 kg at laboratory scale compared to 5 kg or greater at commercial scale (Diefes et al., 1993; Kuo & Gunasekaran, 2003), the contribution of associated packaging is also not often considered.

Freezing impacts on several biochemical processes that occur within the cheese, such as proteolysis. Proteolysis is a critical factor in determining product properties, with increased protein breakdown correlated with cheese softening and adhesiveness (Bertola et al., 1996). However, the effect of freezing on proteolysis is complex. Low temperatures, such as -20 °C, are reported to slow proteolysis in cheese by halting the activity of starter bacteria and any residual coagulant (Fontecha et al., 1994). Proteolysis may be faster after thawing, due to changes in casein conformation induced by freezing and thawing or the lysis of starter bacteria (Fontecha et al., 1994; Verdini et al., 2005). Interestingly, the order of proteolytic pathways remains the same before and after freezing, with α_{s1}-casein being hydrolysed first (Verdini et al., 2005), suggesting that residual coagulant re-activates on thawing. For Mozzarella, both proteolysis and product properties are reported to be optimal after approximately four weeks of refrigerated storage in an unfrozen product (Kindstedt, Carić, & Milanović, 2004), making this a key comparative reference point.

Mozzarella cheese is typically used on a pizza or other baked foods, making its functional properties, including melt and stretch, important to monitor. A further consideration is the duration of frozen storage. Long supply chains can involve extended periods of frozen storage with some evidence that the duration of frozen storage up to nine months can impact on cheese sensory properties for Manchego-type cheese (Prados, Pino, Rincón, Vioque, & Fernández-Salguero, 2006). Previous studies have typically examined cheese stored frozen for up to four months (Fontecha et al., 1996; Oberg et al., 1992). While some studies have extended storage for up to

77 nine months, the focus has been only on sensory and microbiological properties (Prados et al., 2006). Longer
78 studies assessing the impact of freezing could therefore provide new insights.

79 Freezing may also be coupled with tempering, a thawing process performed at refrigeration temperatures.
80 Two weeks of tempering is reported to improve the meltability and stretchability of Mozzarella compared to
81 the freshly thawed product (Kuo & Gunasekaran, 2003; Oberg et al., 1992). This improvement may be due to
82 proteolysis that occurs during tempering (Fontecha et al., 1996), although the exact mechanism is not known,
83 as most studies have not assessed proteolysis under these conditions.

84 The aim of this study was to assess whether chilling and freezing induced structural and functional changes in
85 Mozzarella cheese. The effect of ageing cheese at 4 °C prior to freezing or tempering cheese at 4 °C for one or
86 three weeks after freezing was also determined. The microstructure, proteolysis and functionality of
87 Mozzarella cheese was assessed over a six-month storage period to provide insights to assist with the
88 optimisation of conditions for extended storage.

89 # 2. Materials and methods

90 ## 2.1 Cheese samples and sample preparation

91 A series of 5 kg low-moisture *pasta filata* Mozzarella cheese blocks produced from a single manufacturing run
92 at a commercial cheese factory (Victoria, Australia) were used for trials in order to reduce variability
93 introduced by different manufacturing runs, which was not the focus of this study. The cheese had a standard
94 composition within the range typical for Mozzarella (Kindstedt et al., 2004), with 21.3-21.4% (w/w) fat, 25.1-
95 25.5% (w/w) protein, 49.9-50.4% (w/w) moisture and 0.8-1.0% (w/w) salt.

96 Following manufacture, 36 boxes of cheese were packaged in cardboard and close stacked in 4 layers of 9
97 boxes to form a pallet (Supplementary Information (SI) Figure S1). Each rectangular cardboard box contained
98 4 individually plastic wrapped 5 kg cheese blocks stacked without spacing within the box. Temperature probes
99 (stainless steel immersion food probe attached to Testo 175 T1 – Temperature data logger, Melbourne,
100 Australia) were inserted into selected boxes in the bottom layer (corner box), the second bottom layer

(central box) and third layer (opposite corner boxes). The probe was inserted into the center of one of the four cheese blocks in each of these boxes (see Figure S1). These probe locations were selected to provide an indication of the variability in temperature between blocks in the pallet stacking configuration.

Following packing, the pallet was immediately chilled. Initially there was a rapid chilling phase in a commercial blast freezer with high air velocity at –18 °C for 96 h. After chilling to between approximately 0 °C and -4 °C for 85-95 hours, 14 boxes of cheese were removed from the pallet. These were 9 boxes from the top layer and 5 boxes from the next layer down (Figure S1b). Five of these boxes were transferred to 4 °C storage, where they were refrigerated for up to 6 months to provide control samples. The other 9 boxes were stored at 4 °C for four weeks before transferring to -18°C for up to 5 additional months. The remaining 22 boxes within the pallet (Figure S1c), were further stored and chilled in a -18°C freezer for up to 6 months.

The sample treatments of ageing at 4 °C prior to freezing or tempering at 4 °C after frozen storage were selected as processing and handling steps that industry may employ to supply Mozzarella cheese with optimal functionality. Cheese manufacturers may age Mozzarella cheese prior to freezing or direct retail customers to apply a tempering step to frozen Mozzarella cheese to restore functionality in frozen product.

Frozen samples were collected after one, three or six months of frozen storage at -18 °C and were stored at 4 °C for a further one or three weeks, defined here as tempering, before further analysis. At each timepoint, 2 cardboard boxes were removed from the pallet and each of these cardboard boxes individually placed in 4 °C storage for the tempering process. These conditions reflect a process that often occurs within the supply chain; when product that has been frozen as a pallet at industrial scale may be thawed in smaller packaged units by a retail customer. Refrigerated samples were also collected after one, three and six months of storage at 4 °C. Three of the four 5 kg blocks within each cardboard box were randomly selected for analysis. These storage treatments provided three conditions for comparison: cheese refrigerated at 4 °C, cheese frozen at -18 °C and tempered for one week and cheese frozen at -18 °C and tempered for three weeks. There are also three different time points for comparison for each of the storage treatments with one, three and six months of storage.

In addition, a storage treatment that involved ageing at 4 °C for four weeks followed by frozen storage at -18 °C for five months, to give six months total storage time, was also examined. These samples were used for Synchrotron-Fourier transform infrared (S-FTIR) microspectroscopy, proteolysis analysis and pizza bake tests (see below).

Cheese composition was assessed using the Babcock method (AOAC, 2002) for fat, the Kjeldahl method (IDF, 1993) for total nitrogen, the oven drying method (IDF, 1982) for total solids and the potentiometric method for salt concentration (IDF, 1988). The pH was measured using an Orion 720A pH meter (OrionPacific Pty Ltd, Vic, Australia).

2.2 Microstructure of Mozzarella cheese

The microstructure of cheese samples was analysed by confocal laser scanning microscopy (CLSM; Leica Microsystems, Heidelberg, Germany). Cheese samples of approximately 3 mm x 3 mm x 1 mm in size prepared with a surgical blade were examined along a plane parallel to the aligned protein segments following a published method (Pax, Ong, Kentish, & Gras, 2021). Samples were stained with Nile red and Fast Green FCF (Sigma-Aldrich, St Louis, MO, USA). The Nile red and Fast Green FCF stains were excited at wavelengths of 488 nm and 633 nm, respectively, with emission detected between 520-590 nm and 660-750 nm, respectively.

Three-dimensional (3D) image analysis was conducted using Imaris image analysis software (Bitplane, South Windsor, CT, USA). Each 3D image was constructed from 40 2D images with a gap of 0.25 μm between each layer to give a total sample thickness of 10 μm. Six images were collected for each sample and a representative image is presented.

The cheese samples were also analysed in the direction parallel to the aligned protein strands using cryo-SEM (Quanta, Fei Company, Hillsboro, OR, USA) after six months of storage following an established method (Ong, Dagastine, Kentish, & Gras, 2011). Cryo-SEM images were collected at different magnifications and one representative image is presented.

2.3 Proteolysis of Mozzarella cheese

The protein within the cheese samples was assessed using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), as described previously (Pax et al., 2021). Cheese samples (25 mg) were used to produce stock solutions that were diluted four times with Tris (10 mmol/L)-EDTA (1 mmol/L) pH 8.0 buffer solution. Standards (0.25 mg/mL) of α_{s1}-, β- and κ-caseins (Sigma-Aldrich) were also prepared. Cheese or standard solutions (6.5 μL) were mixed with 2.5 μL Bolt LDS Sample Buffer (4x) and 1 μL Bolt reducing agent (both from Life Technologies, Victoria, Australia) before heating at 70 °C for 10 minutes. The samples and a molecular weight standard (Novex Sharp unstained, Life Technologies) were loaded onto a 4-12% Tris-glycine gel (Life Technologies). The stained gel was scanned using a Chemidoc XRS+ with Quantity One software (Bio-Rad, NSW, Australia). The proteins were identified based on the molecular weight standards. Quantification of gel band intensities was determined using ImageJ (Research Services Branch, Natl. Inst. Of Health, USA). The data presented are the mean ± the standard deviation of three replicates.

The water soluble extract (WSE) of cheese samples was obtained using a previously reported Method (Ong, Henriksson, & Shah, 2006). This extract was used to determine peptide profiles using a modified version of a method described previously (Pax et al., 2021) on an Agilent 1100 Series HPLC (Agilent Technologies, Santa Clara, CA, USA) system fitted with an ultraviolet detector and a 3.6 μm C18 column (250x4.6 mm; Phenomenex, NSW, Australia). The wavelength used for detection was 214 nm. The data presented are the mean ± the standard deviation of three replicates.

2.4 Mozzarella cheese functionality tests

2.4.1 Meltability

Cheese samples 36 mm in diameter and ≈4 mm in thickness were prepared and the meltability assessed using a controlled stress rheometer (AR-G2, TA Instruments, Delaware, USA) with a modified published method (Ma, James, Balaban, Zhang, & Emanuelsson-Patterson, 2013). A temperature sweep test was conducted from 20 °C to 65 °C at 0.05% strain and 0.8 Hz in the linear viscoelastic region. The crossover between the elastic modulus (*G′*) and the viscous modulus (*G″*), defined as the transition temperature, was determined from this test. The data presented are the mean ± the standard deviation of three replicates.

2.4.2 Stretchability

The stretchability of the Mozzarella cheese samples was assessed using an Instron 5944 Single Column Electro-Mechanical Tension Compression Machine (Instron Corp., Norwood, USA) fitted with 2 kN load cell and rubber-coated grips. A mass of 25 g of shredded cheese was melted onto a cracker (Salada; dimensions 9 cm x 10 cm; Arnotts Biscuits Ltd, NSW, Australia) at 240 °C for 3.5 minutes. The cheese cracker was then carefully broken into two halves along the perforated line and secured within the machine grips. The instrument then pulled apart the halves to a total distance of 40 cm and the force required to move the cheese and final length of the melted cheese mass were measured. The data presented are the mean ± the standard deviation of three replicates.

2.4.3 Pizza bake test

A pizza bake test was conducted to assess functionality on a pizza using a pizza base (23 cm in diameter; Coles, Victoria, Australia) that was evenly coated with 90 g of a 1:1 blend of water to tomato paste (Leggos, Victoria, Australia). Shredded Mozzarella cheese (100 g) was evenly dispersed over half of the pizza and the pizza baked at 245 °C for 2:45 minutes in an impinger-style pizza oven (High h Conveyor 2020, Turbochef, Carrollton, TX, USA). These conditions are routinely used within the industry to assess cheese functionality.

The baked pizza was visually evaluated for melted cheese characteristics including: melt, the extent of integration of the separate shreds (from visible shreds to completely melted); free oil release, the volume of fat released on the surface (from none to very high with optimum being the middle point); browning, the colour intensity of the cheese (from light brown to very dark brown); blistering, the elevated areas of melted cheese (including blister size and percentage coverage); and skinning, the extent to which cheese pulls together across the surface using a fork (from very weak to strong skinning). The stretch length was also assessed, but is not reported here, as the instrumental method described in Section 2.4.2 was determined to provide similar, yet more objective, results. With the exception of melt and skinning, a good quality Mozzarella cheese will, by industry convention, have pizza bake results in the middle of the qualitative ranges provided. In the case of melt, complete melting is desirable; while very weak skinning is an indicator of a quality cheese. The results presented are representative of three baked pizzas evaluated by one assessor.

2.5 Synchrotron-FTIR microspectroscopy of Mozzarella cheese

The S-FTIR microspectroscopic analysis was conducted in transmission mode following a previously published protocol (Pax et al., 2019). Thin sections of 8 µm of Mozzarella cheese were prepared in the direction parallel to the protein strands using a cryo-microtome adjusted to -25 °C (Cryostat, Hyrax C60, Carl Zeiss, Germany) and transferred to CaF_2 windows. Data were collected at the Australian Synchrotron Infrared Microspectroscopy (IRM) Beamline (Victoria, Australia) using a Bruker Vertex 80v spectrometer coupled with a Hyperion 2000 FTIR microscope and a liquid nitrogen-cooled mercury cadmium telluride (MCT) detector (Bruker Optik GmbH, Ettlingen, Germany). A matching 36x Schwarzschild IR reflecting objective and condenser (NA = 0.5) were used for data acquisition with a beam aperture size of 6.9 µm in diameter and step interval of 3 µm. All S-FTIR spectra were recorded within a spectral range of 3800-700 cm^{-1} using 4 cm^{-1} spectral resolution. Blackman-Harris 3-Term apodisation, Mertz phase correction and zero-filling factor of 2 were set as default acquisition parameters using the OPUS 7.2 software suite (Bruker).

FTIR maps of Mozzarella cheese stored at 4 °C for six months, -18 °C for six months, -18 °C storage for six months followed by tempering at 4 °C for three weeks and aged at 4 °C for four weeks followed by frozen storage at -18 °C for five months were acquired within a 60 µm x 60 µm area, resulting in a total of 400 spectra per sample. Two cheese samples were analysed per storage treatment with representative FTIR intensity maps and average spectra presented.

After spectral data acquisition, atmospheric compensation was applied using OPUS 7.2 software suite (Bruker) to remove interference from atmospheric water vapour and carbon dioxide from the spectra. The resultant spectra were then pre-processed in CytoSpec v. 1.4.02 software (Cytospec Inc., Boston, MA, USA) using a noise reduction algorithm before conversion into second derivative spectra using the Savitzky-Golay (1964) algorithm. Hierarchical cluster analysis (HCA) was subsequently performed to sort the pre-processed second derivative spectra into five clusters based on their similarity in the two spectral ranges containing biological bands associated with lipids and proteins (i.e. 3015-2810 cm^{-1} and 1785-1110 cm^{-1}). Here, only clusters containing spectra with high protein to fat ratio were selected for further analysis. The spectra were also processed by applying an extended multiplicative scatter correction (EMSC) algorithm based on the same

spectral ranges containing biological bands associated with lipids and proteins (i.e. 3015-2810 cm^{-1} and 1785-1110 cm^{-1}). After that, principal component analysis (PCA) was conducted using The Unscrambler® 10.1 software package (CAMO Software AS, Oslo, Norway), as described previously (Pax et al., 2019), based on the biological regions between 3000-2800 cm^{-1} and 1800-1100 cm^{-1}, as well as the amide I protein region alone (1700-1600 cm^{-1}).

2.6 Statistical analysis

Statistical analysis was completed with Minitab 17 statistical software (Minitab Inc., State College, PA, USA). All quantitative results were analysed using a "General Linear Model," with significant changes due to time or the storage format being further analysed using Tukey's paired comparison with a significance level of P = 0.05.

3 Results and discussion

3.1 Heat transfer during freezing of Mozzarella cheese

The temperature of the 5 kg Mozzarella block samples packed within a pallet, during blast chilling and freezing, was monitored to provide an estimate of the rate of heat transfer and length of time needed to cool and freeze 5 kg Mozzarella blocks under industrial conditions. In this study, the four selected 5 kg blocks took 657-972 hours to reach -17 °C from an original temperature of 21-23 °C (for further details see Supplementary Information). Once frozen, the cheese temperature remained relatively constant at -17 °C for the duration of the storage period, with only small fluctuations of 1-2 °C, as occurs during industrial storage.

The time taken to freeze cheese may be defined as the length of time needed to cool from -1.1 °C to -6.7 °C (Bertola et al., 1996; Diefes et al., 1993). Using this definition, the 5 kg Mozzarella cheese blocks took 114 $\pm$ 20 h to freeze under the industrial conditions applied here. This rate of freezing is at least 9 times slower than the equivalent rate of ~10 h reported under laboratory conditions (Bertola et al., 1996), as shown in Table 1. It is also approximately eight times slower than the rate of cooling observed in a recent study where three different rates of cooling for Mozzarella cheese were examined (To et al., 2020), where the slowest freezing rate was 0.6 °C h^{-1} for a 2.5 kg block. Factors contributing to the slower heat transfer rate measured here

include the size of the cheese blocks (5 kg vs 1.5-2.5 kg in previous studies) and the packaging and the pallet configuration, which slows heat transfer relative to a single air-chilled block. Other factors include the freezer temperature (-18 °C), which results in a slower heat transfer than rapid freezing in liquid nitrogen (-196 °C), which may be employed at a laboratory scale (Oberg et al., 1992) and may be more relevant for other methods of freezing such as IQF. The cheese within the 5 kg blocks froze at -1.65 $\pm$ 0.06 °C, using a definition of the temperature at the start of the short plateau that occurs directly after supercooling (see inset Figure S2). This freezing temperature is similar to previous reports, which range from -0.5 °C to -1.7 °C for Mozzarella of a similar composition (Kuo & Gunasekaran, 2003; Ribero et al., 2007).

The extent of supercooling, defined as the difference between the freezing temperature and the lowest temperature reached before freezing, was 3 °C or less, similar to previous studies of Mozzarella cheese (Ribero et al., 2007). Shorter supercooling periods are reported to decrease ice crystal size (Cheng, Sun, Zhu, & Zhang, 2017) and may have led to the formation of smaller ice crystals here.

The four temperature probes placed within select 5 kg Mozzarella blocks indicate that while the slow rate of heat transfer was highly similar between 5 kg blocks, relative to other previous studies, there is some variability in cheese temperature between blocks as a result of block placement under industrial conditions. The box at the bottom of the pallet cooled fastest (green; Figure S1a) and box in the centre of the pallet cooled slowest (red; Figure S1a). Consequently, the time taken for freezing, i.e., for the 5 kg blocks to cool from -1.1 °C to -6.7 °C varied between 175 hours for the box at the bottom of the pallet to 252 hours for the box in the centre of the pallet, although all boxes reached a similar temperature of -12 °C by 300 hours.

At present there is no simple and effective method for predicting the time taken for food products, such as cheese, to freeze or thaw (Góral, Kluza, Spiess, & Kozłowicz, 2016). The data presented here on chilling, freezing and cooling may be useful, however, in the calculation of parameters describing heat transfer in cheese using a so-called inverse problem approach (Ramm, 2005).

3.2 The effect of freezing and tempering on the microstructure of Mozzarella cheese throughout storage

The parallel alignment of fat and protein observed here by confocal laser scanning microscopy is characteristic of Mozzarella cheese (Figure 1) and was clearly visible in 5 kg block samples that had been frozen for either 1 , 3 or 6 months regardless of the length of tempering applied (Fig. 1e-g and i-k). This structure is similar to the structure observed in samples stored at 4 °C for only one month (Fig. 1a) but significantly different to the structure of the cheese aged at 4 °C for longer periods, where there was a loss of protein and fat alignment due to proteolysis (for example, Fig. 1c). The microstructure images therefore indicate that the structural alterations that normally occur due to proteolysis during refrigerated ageing (Fontecha et al., 1996) were prevented by freezing in the present study. The increased structural homogeneity that occurs within Mozzarella stored at 4 °C was particularly evident at six months here, similar to observations of structural rearrangement in cheese stored at 4 °C for shorter time periods (3-6 weeks) (Banville, Morin, Pouliot, & Britten, 2013; McMahon, Fife, & Oberg, 1999).

Importantly, the freezing conducted here did not appear to induce large cracks or other visible defects, a result confirmed by cryo-SEM of the samples frozen for six months (Fig. 1d, h, l). The absence of visible defects was potentially due to the slow rates of freezing and thawing applied. Pockets of fat globules aligned between protein were visible in 5 kg blocks stored at -18 °C, similar to confocal images, whereas the cheese blocks stored at 4 °C contained a more homogeneous distribution of protein. These refrigerated samples showed a greater susceptibility to artefacts which can be introduced by cryo-SEM sample preparation after six months of storage, including webbing or crystalline structures present within the protein network (see Fig. 1d, red dashed arrows). These potentially arise from the softer nature of these cheese blocks. There was some evidence of minor cracks in selected cheese samples that were stored frozen and tempered for one week (see Fig. 1h, white arrow), however, these cracks were not systematically observed across multiple samples.

The microstructural data suggest that the slow freezing rate of the industrial conditions applied here, where it takes 84-130 h to cool the sample from -1.1 °C to -6.7 °C, causes less product damage than the conditions used in laboratory experiments, where freezing induced damage to the cheese protein matrix was detected.

The cracks observed previously were 40 µm to >100 µm in length and 2-5 µm in width. Two studies used much faster freezing rates between 1.5 h and 4 h to cool from -1.1 °C to -6.7 °C (for further information see Table 1). Another study observed small cracks up to 10 µm in length and 2-3 µm in width (Ribero et al., 2009) but did not report the conditions or rates of heat transfer (Fontecha et al., 1996; Kuo & Gunasekaran, 2009; Ribero et al., 2009). It is worthy of mention that these past studies used SEM to evaluate structural damage, rather than the cryo-SEM employed here and this may be a further factor in the differences observed.

The structural damage observed on freezing was attributed to the formation of large ice crystals inducing mechanical stresses on the cheese matrix during freezing (Fontecha et al., 1996; Kuo & Gunasekaran, 2009). Although, it is possible that the cracks were caused by the contraction in overall cheese volume that can occur upon rapid cooling. Cracks of these scale, however, are unlikely to affect the functional properties and quality of commercially produced cheese (Ribero et al., 2009).

3.3 The effect of freezing and tempering on proteolysis in Mozzarella cheese during storage

The effect of freezing and tempering on proteolysis within the 5 kg cheese blocks was examined by SDS-PAGE and RP-HPLC. While frozen storage is expected to halt biochemical changes such as proteolysis (Prados et al., 2006; Sharma, Su, Joshi, Roux, & Sathe, 2010), processes such as bacterial cell lysis may occur with freezing and can accelerate protein breakdown (Verdini et al., 2005).

Proteolysis was not enhanced by blast chilling and was arrested with frozen storage. The breakdown of α-caseins and β-casein was instead determined predominantly by the length of time the samples spent at 4 °C (Fig. 2). Comparison between cheese treatments was found to be more meaningful when plotted by the time spent in the tempering process at 4 °C (Fig. 2a2, a3, b2), rather than as a function of the length of frozen storage (see Figure S3). There was no significant difference ($P > 0.05$) between samples stored at -18 °C for one, three or six months under these conditions, confirming that frozen storage arrested proteolytic breakdown. As expected, the concentration of α- and β-caseins in control samples decreased with storage at 4 °C, reducing significantly ($P < 0.05$) from 45 $\pm$ 5% to 9 $\pm$ 2% for α-caseins and from 34 $\pm$ 2% to 20 $\pm$ 3% for β-

328 casein, with 84% and 90% of these changes occurring within the first three months, respectively
329 (Fig. 2a2-3).

330 Longer tempering induced changes in proteolysis. The concentration of α-caseins was not significantly altered
331 ($P > 0.05$) with longer tempering, with ~45 ± 4% of α-caseins remaining after one week of tempering or 39 ±
332 5% of α-caseins remaining after three weeks of tempering (Fig. 2a2). However, the level of β-casein was
333 significantly lowered ($P < 0.05$) with longer tempering, with 31 ± 1% of β -casein remaining after one week of
334 tempering or 27 ± 2% of β -casein remaining after three weeks of tempering (Fig. 2a3).

335 Ageing cheese samples prior to frozen storage led to the expected increase in proteolysis when compared to
336 frozen samples without the ageing step. The level of α-caseins was 35 ± 8% for aged and frozen samples that
337 were tempered for one week, compared to 28 ± 4% for aged and frozen samples tempered for three weeks.
338 Similarly, the β –casein concentration in aged and frozen samples tempered for one week was 32 ± 4% and 29
339 ± 6% for cheese samples aged and frozen and tempered for three weeks.

340 The peptide profiles determined by HPLC analysis also confirmed that proteolysis was halted with frozen
341 storage. Profiles extracted for cheese frozen for six months and then tempered for one or three weeks show
342 significantly fewer peaks than cheese refrigerated for six months, with cheese that was aged and then frozen
343 having a number and level of peaks in between the other two treatments (Fig. 2b1). These data are best
344 illustrated as a function of time spent at 4 °C (Fig. 2b2) rather than as a function of total time (see Figure S3b).
345 The total peak area in the frozen and tempered samples was also similar ($P > 0.05$) to what might be expected
346 for cheese refrigerated for only a few weeks (Fig. 2b2), with a total area of 17 ± 1.0 Au.min and 18.6 ± 0.6
347 Au.min, respectively, compared to 16.6 ± 0.7 Au.min at four weeks for the refrigerated sample at the earliest
348 measurement point. Again, the length of frozen storage (one, three or six months) did not appear to
349 significantly impact proteolysis, as determined by the total peak area.

350 There was an approximately 1.7-fold increase in total peak area determined by HPLC over the six months of
351 refrigerated storage (Fig. 2b2), while the decrease in α-caseins for the same period was ~2.5-fold (Fig. 2a2).
352 The literature typically reports an approximately two-fold increase in soluble nitrogen, an alternative measure

353 of proteolysis, after two to three months of storage (Costabel, Pauletti, & Hynes, 2007), similar to the data
354 reported here.

355 Accelerated proteolysis, because of freezing and tempering, was not observed here. As expected, proteolysis
356 appeared to occur during the time the samples spent at 4 °C, including tempering, with the length of time
357 spent at 4 °C influencing casein breakdown, potentially enabling the effect of tempering to be predicted and
358 optimised under these conditions. These data confirm that industrial freezing halts biochemical changes even
359 when the rate of chilling and freezing is very slow. The data also show that proteolysis is halted when the
360 samples are stored frozen for up to six months. Prior studies have reported an increase in the rate of
361 proteolysis after frozen storage and these changes have been attributed to structural damage or to
362 conformational changes in the protein network, where proteolysis was observed to increase after 13-15 days
363 (Fontecha et al., 1994; Verdini et al., 2005). Here, the slower rate of chilling and freezing and the absence of
364 structural damage observed (Fig. 1) by CLSM and cryo-SEM may have minimised any protein breakdown.

365 ## 3.4 Functionality of Mozzarella cheese during storage

366 The process of freezing and the duration of frozen storage did not impact significantly on either meltability or
367 stretchability, two functionality tests important for industry application. Quantitative methods were applied
368 to assess functionality: the meltability of Mozzarella cheese was assessed by measuring the transition
369 temperature, defined as the temperature at which cheese begins to flow as measured in a rheometer and the
370 stretchability of Mozzarella cheese was assessed using the stretch length, defined as the maximum length that
371 the cheese can extend under tensile load before the strands break.

372 The duration of tempering (one or three weeks) affected the meltability of Mozzarella cheese. The data are
373 presented here as a function of time spent at 4 °C (Fig. 3), as the comparison is more meaningful than when
374 plotted as a function of total time (Figure S4). Samples frozen and tempered for one week had a transition
375 temperature of ~53 °C regardless of frozen storage time, whilst samples frozen and tempered for three weeks
376 had a significantly lower ($P < 0.05$) transition temperature of ~49 °C, similar to the transition temperature of
377 48.7 °C measured for Mozzarella refrigerated at 4 °C for four weeks (Fig. 3a). This observation is consistent
378 with proteolysis occurring predominantly during the time samples spent at 4 °C. As expected, the meltability

379 of the Mozzarella increased significantly ($P < 0.05$) with longer storage at 4 °C, as shown by the decrease in
380 transition temperature from 48.7 °C at one month to 41.5 °C at six months (Fig. 3a), correlating with the
381 increased proteolysis that occurred in these samples (Fig. 2).

382 The stretchability was also affected by tempering duration. The stretch was long (40 cm) for samples
383 tempered only one week. Longer tempering greatly increased the variability in the stretch length measured,
384 making comparison with these samples more difficult, although the stretch length included a range similar to
385 cheese refrigerated for an equivalent length of time (Fig. 3b). The stretch length was significantly lower ($P <$
386 0.05) for samples refrigerated for longer periods, measuring 27 ± 1 cm for cheese refrigerated for one month
387 and then 10 ± 2 cm for cheese refrigerated for six months, again consistent with the greater proteolysis that
388 occurred in these samples (Fig. 2).

389 The increase in proteolysis with longer tempering, as shown by the change in β-casein in Fig. 2a3, likely
390 contributed to the significant changes observed in both transition temperature and stretchability, as protein
391 networks with fewer intact proteins are expected to flow more easily at lower temperatures and to have
392 reduced stretch under tensile load. The greater rehydration of the protein network during tempering is a
393 further factor that could contribute to the observed changes. Freezing is proposed to dehydrate the protein
394 network (Diefes et al., 1993), making proteins more compact and more strongly interactive (Diefes et al.,
395 1993; Ribero et al., 2009). Tempering may allow the protein network time to hydrate, as the time spent at 4
396 °C after frozen storage has been shown to reduce the more-mobile serum fraction to near 0 after 15 days,
397 similar to the protein network hydration that occurs in Mozzarella cheese directly after manufacture at 4 °C,
398 where proteins absorb serum (To et al., 2020) and the free water in this serum associates with the casein
399 network (Kuo, Gunasekaran, Johnson, & Chen, 2001; Smith, Vogt, Seymour, Carr, & Codd, 2017). Changes to
400 protein hydration will alter the size, shape and structure of the protein at a molecular scale, potentially
401 altering the response of the protein network to heat, shear and tensile load. Further studies could seek to
402 assess the impact of protein hydration on these properties, although it may be difficult to isolate and assess
403 the impact of hydration independently of other factors in such a complex matrix.

A key observation in the present study is that tempering Mozzarella cheese for three weeks provides a functionality similar to that of unfrozen cheese stored for one month. Tempering for longer periods may also be responsible for the improvement in meltability and stretchability reported in other studies (Kuo & Gunasekaran, 2003; Oberg et al., 1992). Little systematic improvement in cheese meltability and stretchability was observed for Mozzarella cheese, however, when the duration of the ageing and tempering periods was varied in previous studies (Kuo & Gunasekaran, 2003).

Pizza bake tests are commonly employed as routine quality tests in industry and were conducted here to qualitatively assess cheese melt, colour, free oil and blistering and to allow comparison to the quantitative measures of meltability and stretchability (see Table S1) and translation to industry practice.

Both freezing and the duration of frozen storage influenced some measures of functionality in these tests. Freezing increased skinning, an undesirable trait where cheese can hold together and pull across the pizza surface; this was particularly evident in samples frozen for a month but reduced with longer frozen storage.

Tempering for three weeks improved several pizza bake properties compared to tempering for one week. Meltability increased for samples tempered for three weeks, defined as the visible loss of shred shape or identity on the pizza, which is a desirable trait. Cheese that was aged prior to freezing also had increased meltability compared to frozen samples tempered for one week. Tempering for three weeks also increased the diameter of the blisters formed in frozen samples to a qualitatively more acceptable level.

Cheese that had been aged at 4 °C prior to frozen storage maintained acceptable pizza bake properties, with similar acceptability overall to cheese that had been frozen and tempered for three weeks. The control cheese stored at 4 °C also retained acceptable melt and colour properties throughout the six months of refrigerated storage as expected, with only free oil and blistering appearing less acceptable after six months.

Several factors may influence the changes observed in pizza bake properties with freezing and tempering, although it should be noted that the mechanisms underlying changes in the traits described in the pizza bake tests are complex and not well understood. The changes induced by tempering are likely related to proteolysis, as discussed for melting as well as stretch length above. Proteolysis may alter the mobility of fat

429 in the cheese network, as many of the properties in the pizza bake tests relate to the behaviour of fat. The
430 changes in protein hydration induced by freezing could also impact on skinning, as dehydration has also been
431 observed to induce milk skin formation during drying (Kentish, Davidson, Hassan, & Bloore, 2005). The
432 observations made here are also consistent with a previous study where the pizza bake performance was
433 improved when frozen cheese was tempered for a longer period (35 days rather than 16 days; To et al., 2020)
434 The ability to probe the structure of these proteins at a molecular scale within the cheese may provide further
435 insight into the changes observed in these quantitative and qualitative measures of functionality.

436 3.5 Synchrotron FTIR microspectroscopy of Mozzarella cheese

437 S-FTIR microspectroscopy, used to assess the structure of protein within the Mozzarella cheese samples,
438 indicated that protein structure was altered by the process of freezing and tempering conditions under the
439 conditions examined here. Cheese samples stored at 4 °C or -18 °C for six months were assessed alongside
440 cheese stored at -18 °C followed by tempering at 4 °C for three weeks and cheese that was aged for four
441 weeks at 4 °C prior to -18 °C storage.

442 In all samples, S-FTIR spectra contained characteristic bands associated with the key components of
443 Mozzarella cheese, including proteins, lipids and water (Fig. 4a). The bands in the range 3000-2800 cm^{-1} are
444 attributed to the *ν*(C-H) stretching vibrations of methyl ($-CH_3$) and methylene ($-CH_2$) groups within both
445 proteins and lipids. The sharp *ν*(O-H) band at 3600-3100 cm^{-1} represents the O-H bonding interactions of the
446 small number of water molecules remaining within the dehydrated thin sections of cheese. At the lower
447 wavenumber region, the peak at ~1750 cm^{-1} is attributed to *ν*(C=O) stretching of lipid and fatty acid ester
448 functional groups and can be used to represent total lipids (Vongsvivut et al., 2012). The pronounced bands at
449 ~1650 and 1550 cm^{-1} are assigned to the amide I and II modes of proteins respectively. The amide I (1700-
450 1600 cm^{-1}) band is commonly used to characterise protein secondary structure, as the vibrations are directly
451 linked to the backbone conformation, with each frequency denoting a particular secondary structure (Yang,
452 Yang, Kong, Dong, & Yu, 2015).

453 The amide I protein band was used here to uncover differences in protein conformation in Mozzarella cheese
454 that underwent different storage conditions. The second derivative of the amide I peak (Fig. 4b) contained

three main peaks of varying intensities in all samples; at ~1650 cm^{-1} corresponding to α-helical structures, at ~1662 cm^{-1} corresponding to β-turn structures and at ~1639 cm^{-1} corresponding to β-sheet structures (Chen & Irudayaraj, 1998).

FTIR intensity maps of the amide I bands were generated to visualise the distribution of β-turn, α-helix and β-sheet conformations in the Mozzarella cheese samples (Fig. 4c). A higher intensity of α-helical conformation was observed qualitatively in cheese stored at 4 °C for six months, as well as β-turn and β-sheet conformations compared to other samples.

Principal component analysis (PCA) applied to the biological regions at 3000-2800 cm^{-1} and 1800-1100 cm^{-1} uncovered differences in the molecular structure of Mozzarella cheese that underwent different storage conditions. Cheese samples that were aged and then frozen were clearly separated from both frozen and refrigerated samples along the PC1 axis (Fig. 5a), primarily due to variations in the lipid and protein peaks occurring between 3000-2800 cm^{-1}, and also α-helical conformations within amide I protein at 1653 cm^{-1} and amide II protein at 1545 cm^{-1} (Fig. 5b). Along the PC2 axis, which represents 21% of the variation, frozen samples were separated from refrigerated and from both frozen and tempered and aged and frozen samples due to lipid and protein peaks between 3000-2800 cm^{-1} and 1760 cm^{-1} and random coil structures at 1640 cm^{-1}. Similar separation was observed when only the amide I region (1700-1600 cm^{-1}) was used for the PCA, with the key difference being that cheese stored at 4 °C for six months was not differentiated from cheese frozen and tempered at 4 °C for three weeks (Fig. 5c). Here, the difference between cheese that had spent time at 4 °C and frozen stored cheese was due to β-sheet and β-turn structures, which accounted for 5% of the variation.

These data are significant, indicating that at least partial recovery of protein structures is possible in frozen and tempered samples so that they are indistinguishable from samples stored at 4 °C in the amide I region by PCA. This recovery is consistent with prior observations that tempering partially restored cheese properties (Kuo & Gunasekaran, 2003). The recovery of cheese during refrigerated tempering appears to arise from a decrease in β-sheet and β-turn structure induced during frozen storage. β-sheet and α-helical protein structure have previously been shown to correlate to food texture in aged cheese or in meat. Specifically, the

α-helical content of semi-hard ewe's milk cheese was reported to decrease and β-sheet structure increase with age, suggesting these structural changes correlate with a softer overall texture (Fontecha, Bellanato, & Juarez, 1993). A tougher or firmer texture was also found in meat muscle with higher levels of α-helical protein conformation (Warner et al., 2022). In this case, however, the structural changes appeared reversible.

Ageing cheese samples prior to freezing also altered the secondary structure in samples that were then frozen and thawed (Fig. 5c), resulting in samples which were different to those tempered after frozen storage. This observation provides new support for the widely studied structural rearrangement that occurs in *pasta filata* Mozzarella cheese during the initial weeks following manufacture (Kindstedt et al., 2004; McMahon et al., 1999; Smith et al., 2017). Prior work has generally observed that any combination of ageing and tempering results in a cheese with acceptable functionality and texture (Kuo & Gunasekaran, 2003). The FTIR microspectroscopic techniques employed here, however, suggest that tempering after frozen storage is more effective at restoring protein structures. The extent of proteolysis was similar in cheese samples aged for four weeks or tempered for three weeks. These measures indicate that the altered protein structure observed by S-FTIR microspectroscopy are able to distinguish between cheese that has undergone ageing prior to freezing and cheese that has been tempered after frozen storage.

4 Conclusions

Mozzarella cheese blocks (5 kg) were blast chilled and froze slowly over ~114 hours. Under these industrial conditions and in combination with a tempering step of one or three weeks, frozen storage did not lead to cracks or visible changes to the protein network. Ageing cheese for four weeks prior to frozen storage also did not lead to protein network damage. The duration of frozen storage had little impact on the cheese microstructure, proteolysis and most measures of functionality (transition temperature and meltability), indicating that cheese can be frozen for extended periods of up to six months. Tempering Mozzarella cheese for three weeks led to functional properties similar to an unfrozen cheese aged for a month; improving meltability, stretchability and pizza bake properties, potentially due to increased proteolysis and greater protein rehydration. The length of tempering may therefore be used to predict or adjust casein breakdown

and optimise functionality after frozen storage. The protein secondary structures were different for cheese that underwent different treatments, highlighting the discriminatory ability of S-FTIR microspectroscopy to separate samples that was not possible with other techniques. This study shows the importance of studying freezing and thawing using industrially relevant conditions, which may provide initial data useful for modelling heat transfer during chilling and freezing. There is potential to also further extend this research to consider changes to the fat within Mozzarella cheese on freezing.

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Author contributions

A. Pax: conceptualisation, methodology, investigation, formal analysis, data curation, writing- original draft, visualisation

L. Ong: investigation, methodology, writing- review & editing

R. Pax: visualisation, formal analysis, writing- review & editing

J. Vongsvivut: investigation, methodology, validation, writing- review & editing

M. Tobin: resources, methodology, writing- review & editing

S. Kentish: validation, writing- review & editing, supervision

S. Gras: conceptualisation, resources, writing- review & editing, supervision, project administration, funding acquisition

Conflict of interest

The authors declare no conflict of interest.

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647 **Table 1: Rates of heat transfer and conditions used to freeze cheese.**

Cheese type	Freezing rate*	Time taken from -1.1 °C to -6.7 °C (h)	Calculated rate of linear heat transfer from -1.1 °C to -6.7 °C (°C/ h)**	Cheese block size	Cheese block weight (kg)	Freezer conditions	Reference
Low-moisture Mozzarella	**Very slow**	**84-130**	**0.07 – 0.04**	**35 x 11.5 x 11 cm**	**5**	**Freezer at -18 °C**	**Current study**
Low-moisture Mozzarella	Slow	3.5	1.6	5 x 10 x 7 cm	1.5	Still air at -21 °C	(Kuo and Gunasekaran, 2003)
Low-moisture Mozzarella	Slow	2.3	2.4	7 x 30 cm cylinders	2.5	Blast freezer at -30 °C	(Diefes et al., 1993)
Low-moisture Mozzarella	Slow	10	0.6	28 x 10 x 10 cm	n.a.	Freezer at -20 °C	(Bertola et al., 1996)
Low-moisture Mozzarella	Fast	0.22	26	28 x 10 x 10 cm	n.a.	Ethyleneglycol bath at -20 °C	(Bertola et al., 1996)
Semi-hard ewe's milk	Slow	1.5	3.7	10 x 21 cm cylinders	3	Plate freezer at -35 °C	(Fontecha et al., 1996)
Semi-hard ewe's milk	Fast	0.45	12	10 x 21 cm cylinders	3	Liquid nitrogen cooled chest freezer at -80 °C	(Fontecha et al., 1996)
Low-moisture Mozzarella	Slow	9.3***	0.6	28 x 10 x 8 cm	2.5	Freezer at -20 °C	(To et al. 2020)
Low-moisture Mozzarella	Slow	2.8***	2	28 x 10 x 8 cm	2.5	Freezer at -40 °C	(To et al. 2020)
Low-moisture Mozzarella	Fast	0.7***	8	28 x 10 x 8 cm	2.5	Freezer at -40 °C	(To et al. 2020)

648 * Where very slow is defined as >10 h, slow is defined as 2-10 h and fast is defined as <2 h from -1.1 °C to -6.7 °C.
649 ** Calculated using a simple linear approximation
650 *** Time taken calculated from the freezing rates of 0.6, 2 and 8 °C/ h provided in To et al. (2020).

Figure captions

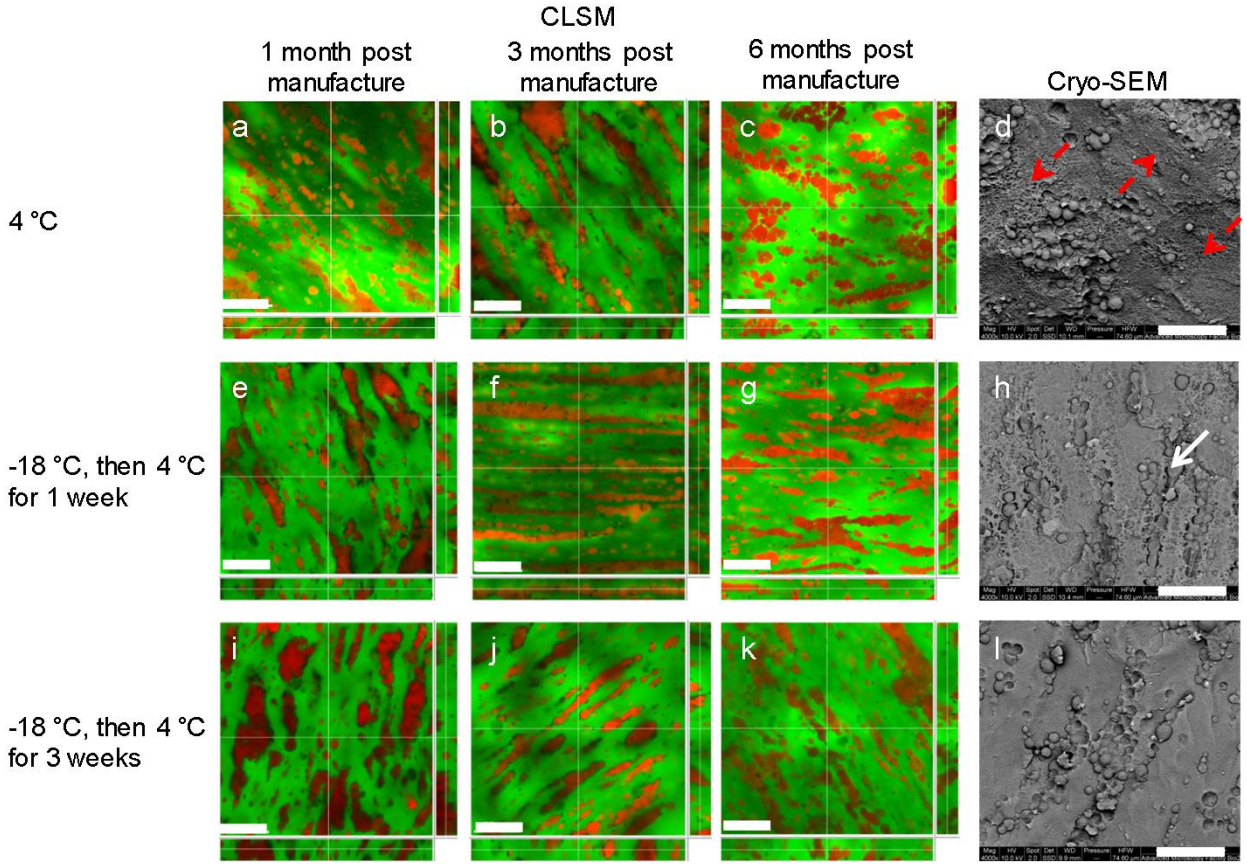
Figure 1: 3D confocal and cryo-SEM micrographs of the microstructure of Mozzarella cheeses stored under three conditions: 4 °C (row 1; a-d), -18 °C and then tempered at 4 °C for one week (row 2; e-h) or -18 °C and then tempered at 4 °C for three weeks (row 3; i-l). Samples were stored for different lengths of time prior to analysis and were examined after one, three or six months of storage by confocal microscopy (columns 1-3 respectively) or after six months by cryo-SEM (column 4). Protein appears green and fat has been stained red in the confocal images. The scale bars for both confocal and cryo-SEM micrographs are 20 μm in length. In the cryo-SEM images, the red dashed arrows indicate webbing in the protein matrix and the white arrow indicates small potential cracks in the cheese microstructure.

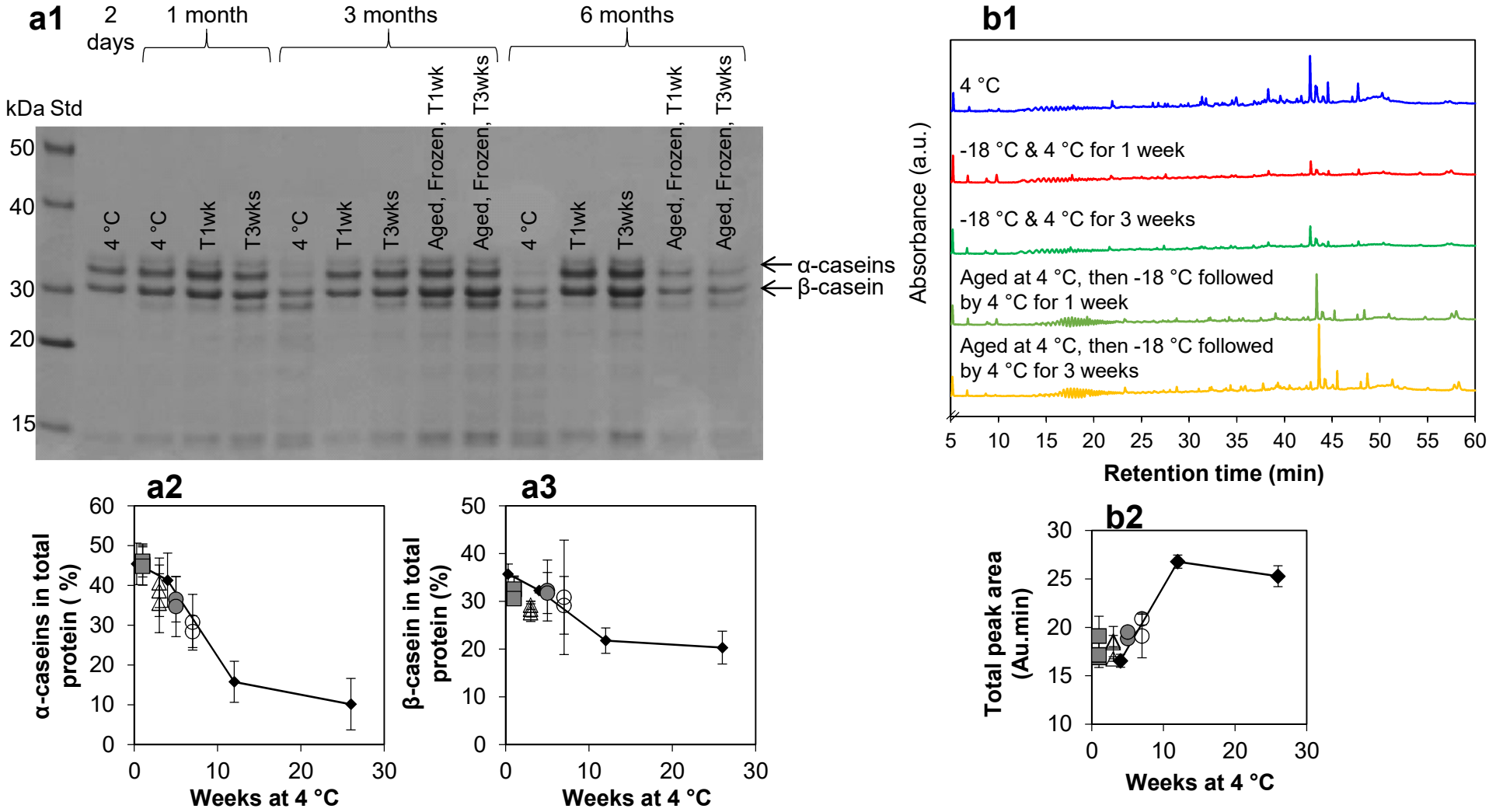
Figure 2: Image of the SDS-PAGE profile (a1) and the change in casein content as a function of time spent at 4 °C determined by image analysis of the SDS-PAGE bands for α-caseins (a2) and β-casein (a3). The HPLC profile of water-soluble peptide extracts at 214 nm obtained after six months of storage (b1) and the changes to the total peak area measured by HPLC as a function of the total storage time at 4 °C (b1). Mozzarella samples were stored at 4 °C (◆), stored at -18 °C and tempered for one week (■), stored at -18 °C and tempered for three weeks (△), aged at 4 °C for four weeks, stored at -18 °C and tempered for one week (●) or aged at 4 °C for four weeks, stored at -18 °C and tempered for three weeks (○). Storage was for one, three or six months. Error bars are ± the standard deviation of three samples. Data points in (a2), (a3) and (b2) include samples stored for one, three and six months; for cheese stored at -18 °C this data is plotted at one and three weeks on the x-axis, corresponding to the total time these samples spent at 4 °C.

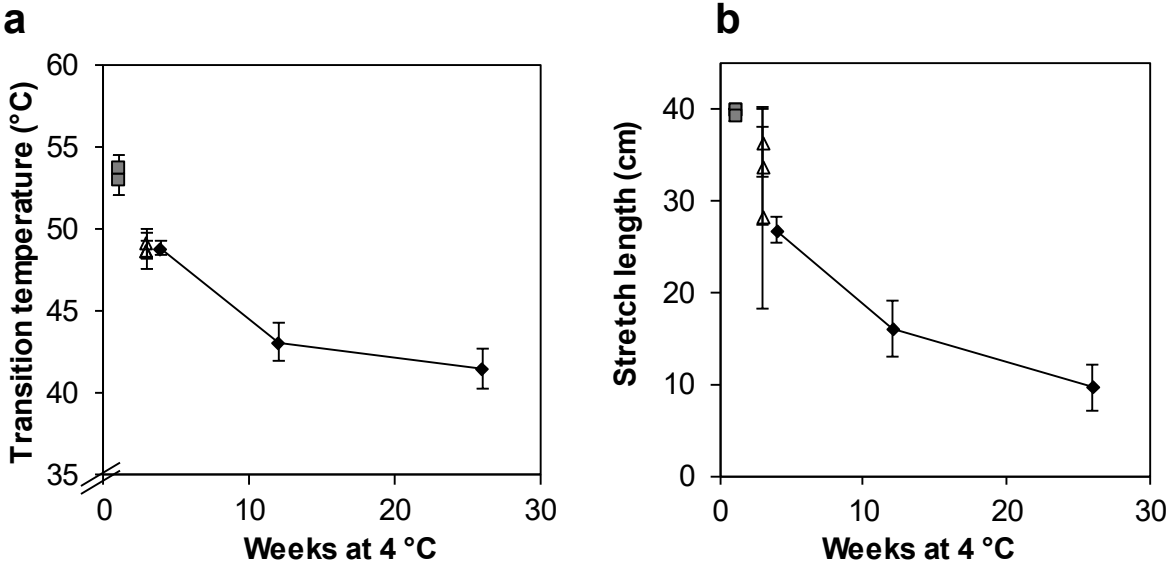
Figure 3: The meltability and stretchability of stored Mozzarella samples. (**a**) Meltability was determined from the transition temperature. **(b)** Stretchability was determined from the stretch length. Mozzarella samples were stored under three conditions: 4 °C (◆), -18 °C and tempered for one week (■), -18 °C and tempered for three weeks (△) for one, three and six months. Data points are the mean ± the standard deviation of three replicates and include data points for samples stored for one, three and six months; for cheese stored at -18 °C this data is plotted at one and three weeks on the x axis, corresponding to the total time these samples spent at 4 °C.

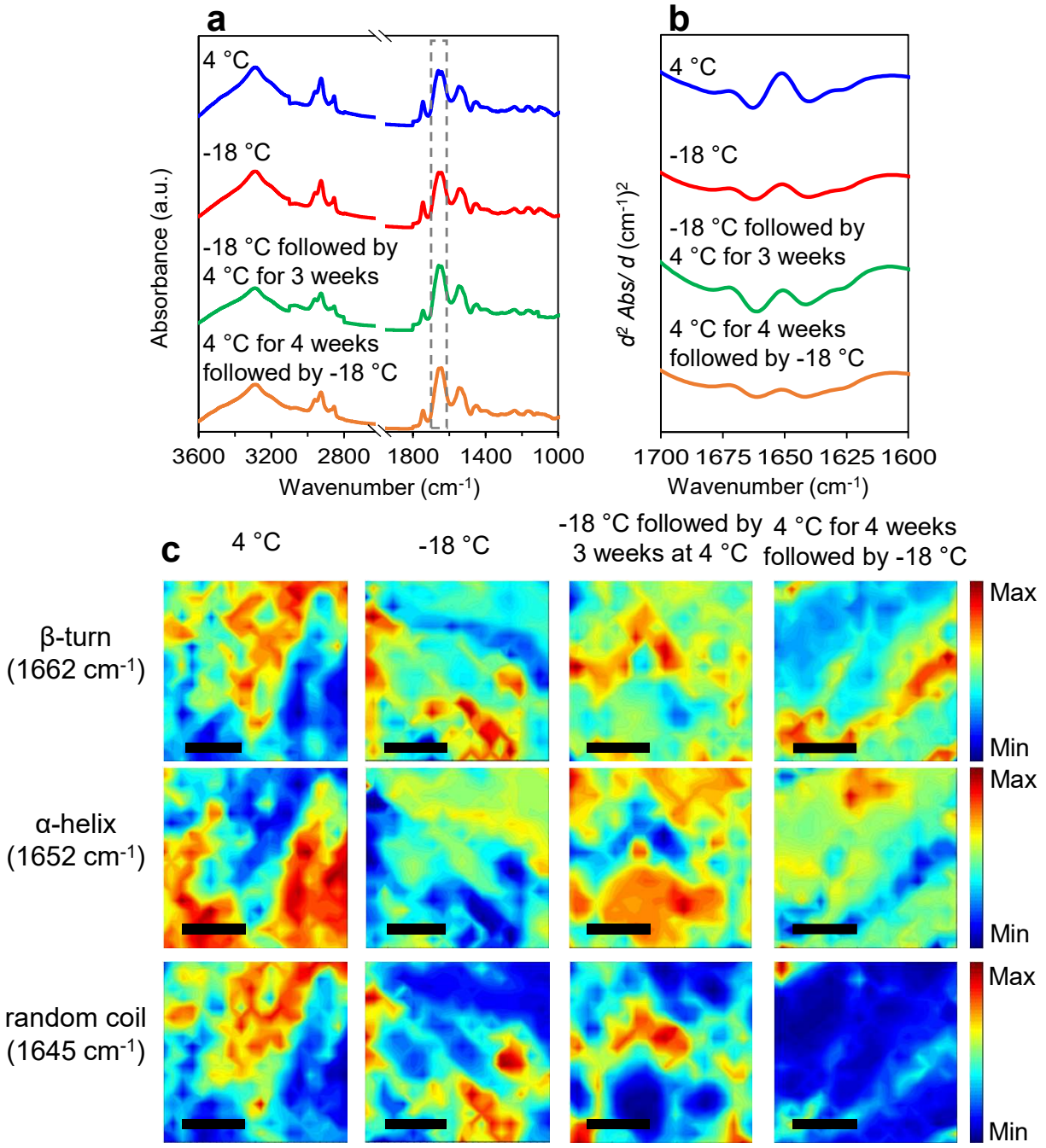
Figure 4: S-FTIR analysis showing the average normalised spectra (a) as determined by HCA. The dashed box in (a) shows the amide 1 region (1700-1600 cm^{-1}) displayed in the second derivative form from areas of high protein in (b). FTIR intensity maps show the spatial distribution of protein secondary structures β-turn, α-helix and random coil for the different Mozzarella cheese storage treatments (c); storage at 4 °C for six months, storage at -18 °C for six months, storage at -18 °C for six months followed by 4 °C for three weeks or aged at 4 °C for four weeks followed by storage at -18 °C for five months. Dark red indicates high structure concentration and dark blue indicates low structure concentration, as signified by the colour intensity scale on the right. The scale bars are 20 μm in length. Representative images are shown for each cheese storage treatment.

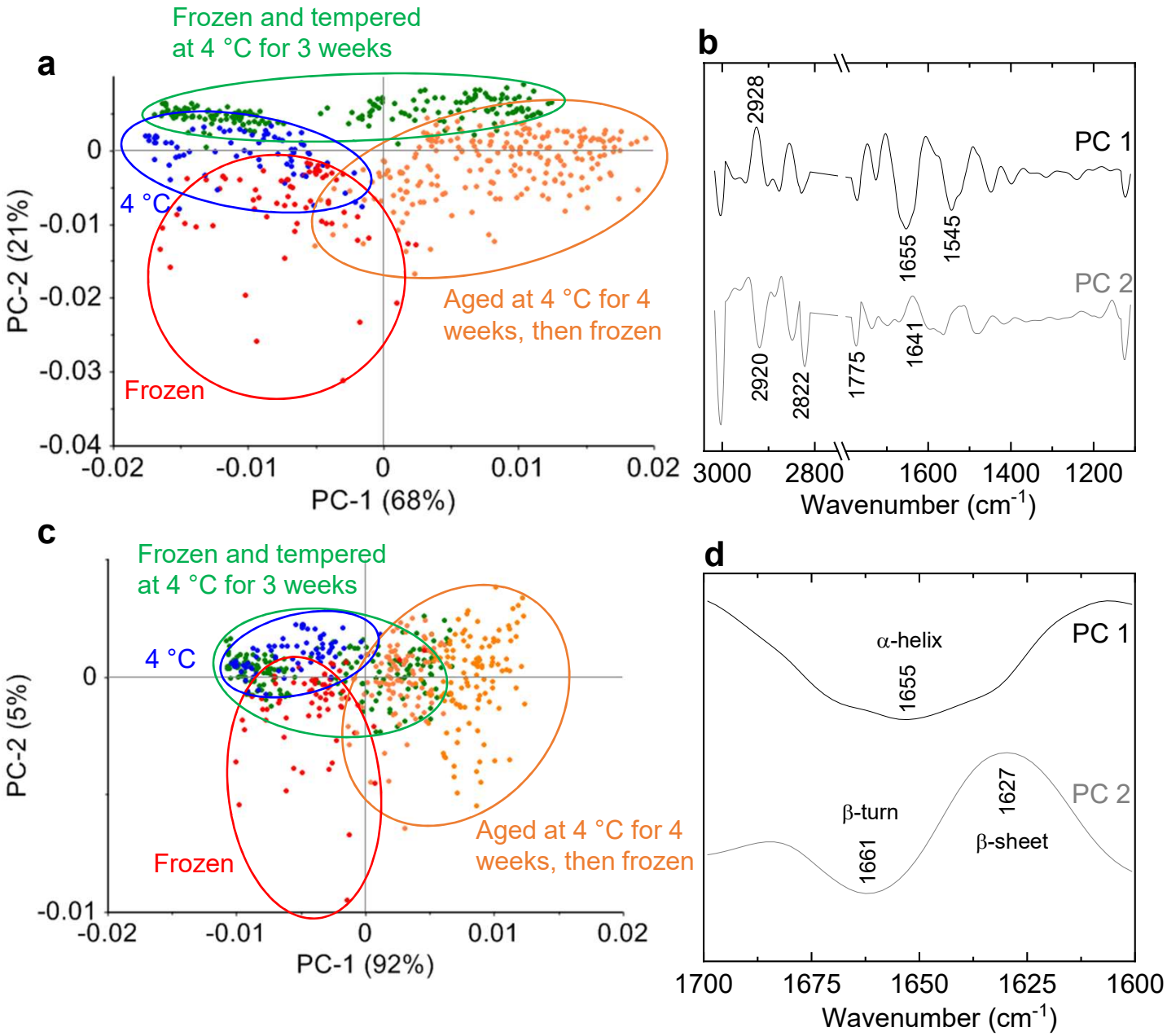
Figure 5: Principal component analysis (PCA) scores (a, c) and loadings (b, d) plots of second derivative high protein spectra in the biological regions between 3000-2800 cm^{-1} and 1800-1100 cm^{-1} (a, b) and the amide I protein region 1700-1600 cm^{-1} (c, d) for cheese stored at 4 °C for six months (blue), -18 °C for six months (red), 4 °C for four weeks then -18 °C for five months (orange) and -18 °C for six months and 4 °C for three weeks (green) samples (n=2).


CLSM
1 month post manufacture
3 months post manufacture
6 months post manufacture
Cryo-SEM
4 °C
-18 °C, then 4 °C for 1 week
-18 °C, then 4 °C for 3 weeks
a
b
c
d
e
f
g
h
i
j
k
l


a1
2 days
1 month
3 months
6 months
kDa Std
50
40
30
20
15
4 °C
4 °C
T1wk
T3wks
4 °C
T1wk
T3wks
Aged, Frozen, T1wk
Aged, Frozen, T3wks
4 °C
T1wk
T3wks
Aged, Frozen, T1wk
Aged, Frozen, T3wks
α-caseins
β-casein
b1
Absorbance (a.u.)
4 °C
-18 °C & 4 °C for 1 week
-18 °C & 4 °C for 3 weeks
Aged at 4 °C, then -18 °C followed by 4 °C for 1 week
Aged at 4 °C, then -18 °C followed by 4 °C for 3 weeks
5 10 15 20 25 30 35 40 45 50 55 60
Retention time (min)
a2
α-caseins in total protein (%)
0 10 20 30 40 50 60
0 10 20 30
Weeks at 4 °C
a3
β-casein in total protein (%)
0 10 20 30 40 50
0 10 20 30
Weeks at 4 °C
b2
Total peak area (Au.min)
10 15 20 25 30
0 10 20 30
Weeks at 4 °C


a
b
Transition temperature (°C)
60
55
50
45
40
35
0
10
20
30
Weeks at 4 °C
Stretch length (cm)
40
30
20
10
0
0
10
20
30
Weeks at 4 °C


a
4 °C
-18 °C
-18 °C followed by
4 °C for 3 weeks
4 °C for 4 weeks
followed by -18 °C
Absorbance (a.u.)
3600 3200 2800 1800 1400 1000
Wavenumber (cm-1)
b
4 °C
-18 °C
-18 °C followed by
4 °C for 3 weeks
4 °C for 4 weeks
followed by -18 °C
d2 Abs/ d (cm-1)2
1700 1675 1650 1625 1600
Wavenumber (cm-1)
c
4 °C
-18 °C
-18 °C followed by
3 weeks at 4 °C
4 °C for 4 weeks
followed by -18 °C
β-turn
(1662 cm-1)
α-helix
(1652 cm-1)
random coil
(1645 cm-1)
Max
Min
Max
Min
Max
Min


a
Frozen and tempered
at 4 °C for 3 weeks
4 °C
Aged at 4 °C for 4
weeks, then frozen
Frozen
PC-2 (21%)
PC-1 (68%)
0
-0.01
-0.02
-0.03
-0.04
-0.02
-0.01
0
0.01
0.02
b
2928
PC 1
1655
1545
PC 2
2920
2822
1775
1641
3000
2800
1600
1400
1200
Wavenumber (cm-1)
c
Frozen and tempered
at 4 °C for 3 weeks
4 °C
Aged at 4 °C for 4
weeks, then frozen
Frozen
PC-2 (5%)
PC-1 (92%)
0
-0.01
-0.02
-0.01
0
0.01
0.02
d
α-helix
1655
PC 1
β-turn
1661
1627
β-sheet
PC 2
1700
1675
1650
1625
1600
Wavenumber (cm-1)

Industrial freezing and tempering for optimal functional properties in thawed Mozzarella cheese

Anita P. Pax[a,b], Lydia Ong[a,b], Randolph A. Pax[c], Jitraporn Vongsvivut[d], Mark J. Tobin[d], Sandra E. Kentish[a], Sally L. Gras*[a,b]

Pallet configuration and temperature probe locations

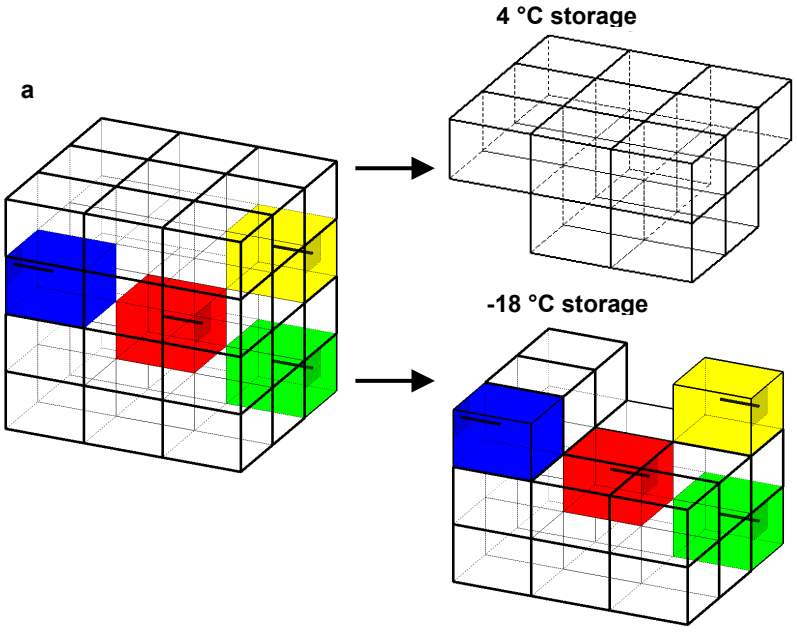


Figure S1: Packing of the 36 blocks of 5 kg Mozzarella cheese within a pallet for chilling and freezing. (a) The 36 boxes of cheese were close stacked in 4 layers of 9 boxes. Probes (indicated by the short black lines) were inserted into boxes in the bottom layer (corner box; green), the second bottom layer (central box; red) and third layer (opposite corner boxes; blue and yellow). (b) After chilling to between approximately 0 °C and -4 °C at 85-95 hours, the top 14 boxes of 5 kg Mozzarella cheese were removed for storage at 4 °C, the remaining 22 boxes of cheese in (c) were frozen and chilled to -18 °C for further storage.

Freezing of cheese

The temperature of the 5 kg Mozzarella block samples stacked within a pallet, during blast chilling and freezing, was monitored using four probes to provide an estimate of the rate of heat transfer and time taken to freeze Mozzarella under industrial conditions (Figure S2). These probes also give an initial

indication of the variability in the temperature measured between selected 5 kg Mozzarella blocks within the pallet.

A number of characteristic heat transfer stages were detected as the 5 kg cheese blocks were chilled and frozen. Initially, cheese at 22.3 ± 1.2 °C was pre-cooled to a temperature above the freezing point. This was followed by supercooling, a process where the cheese is cooled below its freezing point, which continues until there are sufficient nuclei to induce freezing (Ribero, Rubiolo, & Zorrilla, 2007). A phase change then occurs at the freezing temperature, accompanied by a local increase in temperature. This is followed by sub-cooling, where the frozen cheese is further cooled to -18 °C. In this study, these stages occurred over a total time course of 400 hours (Figure S2), although it took a further 590 hours (total of ~990 hours) to reach -18 °C (data not shown in Figure S2). The cheese temperature was fairly constant at -17 °C once frozen, with some small fluctuations that can occur during industrial frozen storage.

The initial data provided here on the variation in heat transfer between four selected 5 kg blocks within the stack of cheese in the pallet suggests that further detailed study of the effect of position in the pallet is needed to fully understand the extent of variation within industrial freezing processes.

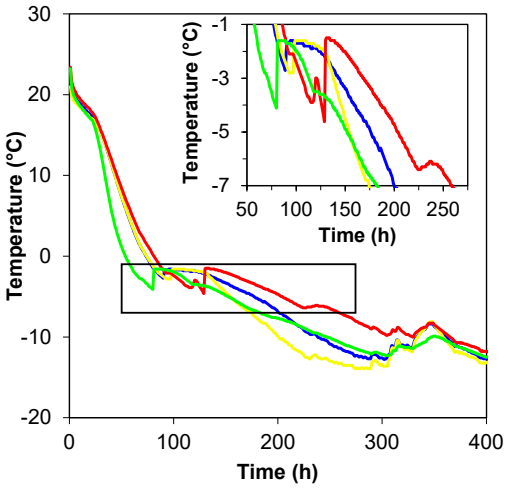


Figure S2: Temperature change within selected 5 kg Mozzarella cheese blocks during chilling and freezing**.** The inset shows the time taken to freeze samples, defined as the time taken to cool from -1.1 °C to -6.7 °C, as well as the point of freezing at -1.65 ± 0.06 °C, defined by the temperature at the start of the short plateau occurring directly after supercooling. The corresponding location of the temperature probes in the four selected 5 kg boxes in (**a**) (green, red, blue and yellow) is shown in Figure S1 above.

Cheese composition and pH

The Mozzarella cheese had a standard composition within the range typical for this cheese (Kindstedt et al., 2004), with 21.3-21.4% (w/w) fat, 25.1-25.5% (w/w) protein, 49.9-50.4% (w/w) moisture and 0.8-1.0% (w/w) salt. The pH of the cheese was 5.3-5.4. No differences in composition were determined between cheese storage treatments.

Proteolysis and functionality measures for thawed Mozzarella cheese

The following figures display the data described in the main text as a function of total storage time (Figures S3-S4). This is in contrast to the figures referred to in the main text, which are plotted as a function of time spent at 4 °C to provide a more meaningful comparison between cheese treatments (Figures 2-3).

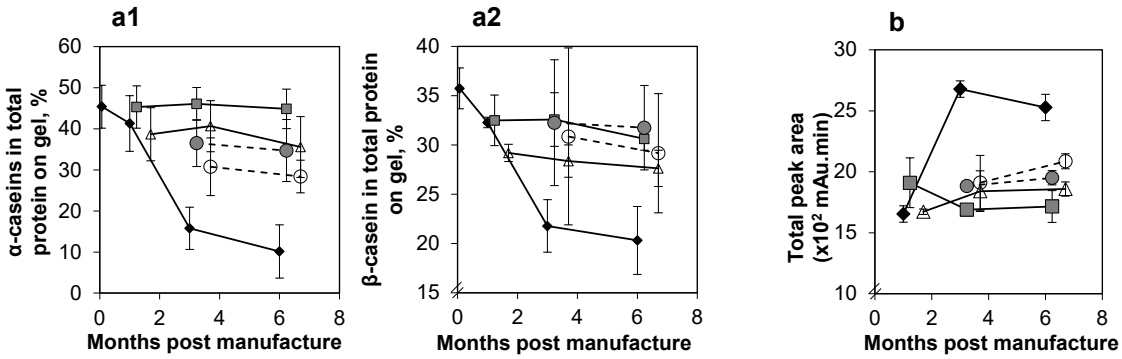


Figure S3: The changes determined by image analysis of the SDS-PAGE gels for α-caseins (**a1**) and β-casein (**a2**). Changes to the HPLC total peak area as a function of storage time (**b**). Mozzarella samples were stored at 4 °C (◆), -18 °C and tempered for one week (■), -18 °C and tempered for three weeks (△), aged at 4 °C for four weeks, stored at -18 °C and tempered for one week (●) or aged at 4 °C for four weeks, stored at -18 °C and tempered for three weeks (○). Storage was for one, three or six months. Error bars are ± one standard deviation of three replicate samples.

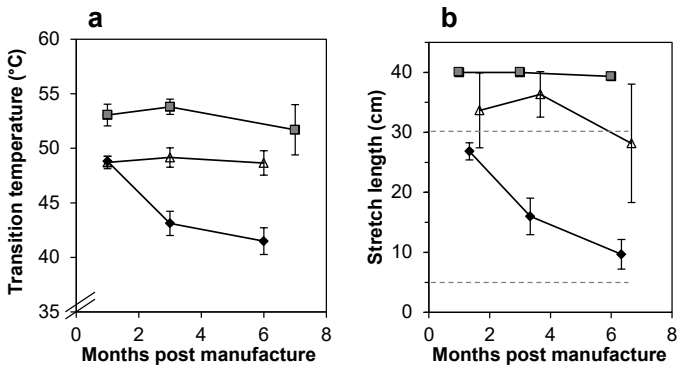


Figure S4: The meltability of Mozzarella samples as measured by the transition temperature determined by rheological analysis (**a**) and stretchability as measured by the stretch length **(b)** for cheese as a function of storage time; stored at 4 °C (◆), stored frozen at -18 °C and tempered for one week (■), stored at-18 °C and tempered for three weeks (△) for one, three and six months. The data presented are the mean ± the standard deviation of three replicate samples.

Table S1: Summary of pizza bake test parameters assessed after one, three and six months of storage. Observations are the average of three pizza bake trials evaluated by one trained assessor.

	1 month post manufacture			3 months post manufacture					6 months post manufacture				
Sample	**4 °C**	**-18 °C then 4 °C for 1 week**	**-18 °C then 4 °C for 3 weeks**	**4 °C**	**-18 °C then 4 °C for 1 week**	**-18 °C then 4 °C for 3 weeks**	**Aged and frozen then 4 °C for 1 week**	**Aged and frozen then 4 °C for 3 weeks**	**4 °C**	**-18 °C then 4 °C for 1 week**	**-18 °C then 4 °C for 3 weeks**	**Aged and frozen then 4 °C for 1 week**	**Aged and frozen then 4 °C for 3 weeks**
Melt	Good	Some outline	Good	Good	Some outline	Good	Good	Good	Good	Good	Good	Good	Good
Skinning	Weak	Strong skinning	Strong skinning	Weak	Strong	Weak	Medium	Weak	No	Medium	Weak	Weak	Weak
Free oil release	Optimum	Optimum	Optimum to heavy	Optimum	Optimum	Optimum	Optimum	Optimum	Low	Optimum to Heavy	Optimum	Optimum	Optimum
Colour	Golden brown	Golden brown	Golden brown	Dark brown	Golden brown	Golden brown	Golden brown	Golden brown	Dark brown	Golden brown	Golden brown	Golden brown	Golden brown
Blistering (diameter)	2-20 mm	2-10 mm	5-20 mm	5-30 mm	2-10 mm	2-20 mm	2-15 mm	2-15 mm	5-30 mm	2-10 mm	5-30 mm	2-25 mm	5-25 mm
(coverage)	25%	20%	20%	35%	20%	25%	20%	20%	30%	25%	25%	30%	30%

References

Kindstedt, P. S., Carić, M., & Milanović, S. (2004). Pasta-filata cheeses. In P. F. Fox, P. L. H. McSweeney, T. M. Cogan, & T. P. Guinee (Eds.), Cheese: Chemistry, Physics and Microbiology (3rd edn, Vol. 2, pp. 251–277). https://doi.org/10.1016/S1874-558X(04)80047-2

Ribero, G. G., Rubiolo, A. C., & Zorrilla, S. E. (2007). Initial freezing point of Mozzarella cheese. *Journal of Food Engineering*, *81*(1), 157–161. https://doi.org/10.1016/j.jfoodeng.2006.10.017