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CICLO XXXII

Statistical, chemometric and computational approaches to improve quality and increase market share of fresh and long-ripened Italian cheeses

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Preface

This PhD thesis conclude a three-years' work carried out in the Technology laboratory of the Food and Drug Department of University of Parma under the supervision of Prof. Germano Mucchetti. Part of this PhD work was carried out in Aarhus University in Denmark in the Department of Food Science, during a four-month stay, under the supervision of Prof. Milena Corredig and Prof. Lars Wiking.

Abstract

Globalization of food markets and the increasing demand for some highly perishable and traditional Italian cheeses, forced the dairy sector to investigate in new methods to improve export and/or to control compliance with PDO regulations or internal quality schemes. Cheese quality characteristics should be improved by studying the strong relation between product's properties and process parameters and efficiency, in order to understand the critical phenomena that are involved in food processing. In this context, chemometric and computational tools are statistical approaches that can be applied to study these complex relations, in particular when many process parameters and product's characteristics are evaluated at the same time, or to model food processing.

Despite its high energy consumption, freezing of fresh dairy products can be a sustainable solution for supply chains: because of the improved storability and convenience, frozen products can be characterized by a lower impact on the carbon footprint if compared to fresh, refrigerated product; cconsidering long-distance markets, freezing can help to lower the costs of transport, by preferring the maritime transport instead of air transport, and can help to decrease waste by decreasing the amount of expired products to be withdrawn from the market. The feasability of high moisture cheese freezing and frozen storage can be valuable, but attention should be payed about process efficiency in terms of energetic costs, and product's sensitivity in relation with the freezing process and frozen storage.

Concerning dairy products regulated by European Protected Designation of Origin (PDO), dairies need fast and reliable methods to control their processes and to verify compliance with both legal limits or more severe internal quality programs. In the case of Parmigiano-Reggiano (P-R) cheese, grated P-R cheese accounted for 13.5% of the overall market of P-R cheese in 2017 and this percentage continuously increased in the last years. Grated P-R PDO cheese must comply with the maximum percentage of rind and particles having a diameter lower than 0.5 mm (<18% w/w and <25%, respectively), because an excessive amount of rind can have a negative impact on the sensory characteristics and product's quality.

The present thesis enabled to improve the knowledge about factors and phenomena that rule the behavior and the modification of fresh and long ripened cheeses during freezing and grating processes.

Mathematical models were created to study Mozzarella cheese freezing process; thanks to a developed photogrammetric technique, it was possible to accurately reproduce Mozzarella cheese non-regular geometry and to accurately estimate freezing time and temperature profiles. Models highlighted the presence of temperature-surface differences related to the irregular geometry of the cheese that can also cause modification of the characteristics in the external layer. Moreover, freezing times were found to be strongly influenced by cheese shape variations. Mathematical freezing models can be valuable in industrial applications to predict freezing times and can be used to control and improve efficiency of Mozzarella cheese freezing process.

Different freezing and thawing rates, that were experimentally modulated as a function of applied air temperature and velocity, did not cause significant modifications of Mozzarella cheese characteristics, as the freezing time was sufficiently short. On the contrary, the presence of covering liquid during freezing and thawing processes caused an absorption of water of Mozzarella cheese, that was found to be more viscous-like and humid than the fresh-one. Thus, freezing in the presence of covering liquid was a less suitable method to freeze Mozzarella cheese.

Frozen storage of Mozzarella cheese slowed down but did not stop proteolytic reactions that showed a higher rate after thawing; oxidative reactions were also present. Moreover, frozen-stored cheeses showed a modification of water status from a molecular and a mesoscopic point of view, as a consequence of casein degradation and conformational change, that caused protein's dehydration. Milk quality and cheese composition should be accurately controlled in order to limit the presence of proteolytic and lipolytic enzymes in the cheese, and to obtain good results after prolonged frozen storage. Moreover, a shorter shelf life period should be proposed for frozen-thawed cheese than the fresh one.

Concerning grated P-R cheese, the grating behavior of different cheeses was found to be related to the textural properties of different zones of the wheel; in particular rind's particles showed different particle size properties and in general were characterized by lower dimensions if compared to particles of the inner part of the cheese. According to these differences, it was possible to successfully predict particle size and rind percentage variations thanks to Near infrared spectroscopy and Image Analysis measurements. These techniques can be successfully applied by dairies as internal quality control measurements to comply with both PDO regulation and quality programs.

Abstract (in italiano)

La globalizzazione dei mercati alimentari e la crescente domanda per alcuni formaggi Italiani tradizionali ed altamente deperibili, ha forzato il settore caseario ad investigare in nuovi metodi per aumentare l'export e/o per controllare la conformità con i disciplinari DOP o con programmi di controllo qualità interni.

Le caratteristiche qualitative del formaggio dovrebbero essere migliorate studiando la forte relazione tra le proprietà del prodotto ed i parametri di processo e la sua efficienza, in modo da capire le criticità che sono osservabili durante la trasformazione dei prodotti. In questo contesto, gli strumenti chemiometrici e computazionali sono approcci che possono essere applicati per studiare queste complesse relazioni, in particolare quando molti parametri di processo e le caratteristiche del prodotto sono valutate contemporaneamente, o per modellare i processi alimentari.

Nonostante l'elevato consumo energetico, il congelamento dei prodotti lattiero-caseari freschi può essere una soluzione sostenibile per le catene di approvvigionamento: grazie al miglioramento della conservabilità e della convenienza, i prodotti surgelati possono essere caratterizzati da un minore impatto ambientale rispetto al prodotto fresco e refrigerato; considerando i mercati a lunga percorrenza, il congelamento può contribuire a ridurre i costi di trasporto, preferendo il trasporto marittimo invece del trasporto aereo, e può contribuire a ridurre gli sprechi diminuendo la quantità di prodotti a fine shelf life da ritirare dal mercato. La potenzialità di congelare formaggi ad alto tenore di umidità ed il loro stoccaggio congelato può essere di grande valore, ma occorre prestare attenzione all'efficienza del processo in termini di costi energetici e alla sensibilità del prodotto in relazione al processo di congelamento e allo stoccaggio congelato.

Per quanto riguarda i prodotti lattiero-caseari regolamentati dalla Denominazione europea di origine protetta (DOP), i caseifici hanno bisogno di metodi rapidi ed affidabili per controllare i loro processi e verificare il rispetto sia dei limiti legali che di programmi di qualità interna più severi. Nel caso del Parmigiano-Reggiano (P-R), nel 2017 il formaggio P-R grattugiato ha rappresentato il 13,5% del mercato complessivo del formaggio P-R e questa percentuale è aumentata continuamente negli ultimi anni. Il formaggio P-R grattugiato DOP deve rispettare la percentuale massima di crosta e particelle con un diametro inferiore a 0,5 mm (rispettivamente < 18% p/p e < 25%), poiché una quantità eccessiva di crosta può avere un impatto negativo sulle caratteristiche sensoriali e la qualità.

Il presente lavoro di tesi ha permesso di migliorare la conoscenza dei fattori e dei fenomeni che regolano il comportamento e la modifica di formaggi freschi ed a lunga maturazione durante i processi di congelamento e grattugia.

Sono stati creati modelli matematici per studiare il processo di congelamento del formaggio Mozzarella; grazie a una tecnica fotogrammetrica sviluppata, è stato possibile riprodurre con precisione la geometria non regolare del formaggio Mozzarella e stimare con precisione i profili di temperatura ed il tempo di congelamento. I modelli hanno evidenziato la presenza di differenze di temperatura sulla superficie, legate alla geometria irregolare del formaggio che può anche causare la modifica delle caratteristiche nello strato esterno. Inoltre, i tempi di congelamento sono stati trovati fortemente influenzati dalle variazioni di forma del formaggio. I modelli matematici di congelamento possono essere preziosi nelle applicazioni industriali per prevedere i tempi di congelamento e possono essere utili per controllare e migliorare l'efficienza del processo di congelamento del formaggio di Mozzarella.

Diverse velocità di congelamento e scongelamento, che sono state modulate sperimentalmente in funzione della temperatura e della velocità dell'aria applicate, non hanno causato modifiche significative delle caratteristiche del formaggio Mozzarella, in quanto il tempo di congelamento era sufficientemente breve. Al contrario, la presenza di liquido di governo durante i processi di congelamento e scongelamento ha causato un assorbimento dell'acqua da parte del formaggio Mozzarella, che è risultato essere più viscoso e umido rispetto a quello fresco. Per questo motivo, il congelamento in presenza di liquido di governo è stato indicato come un metodo meno adatto per congelare il formaggio Mozzarella.

Lo stoccaggio congelato di formaggio Mozzarella ha rallentato ma non ha fermato le reazioni proteolitiche che hanno mostrato un tasso più elevato dopo lo scongelamento; erano inoltre presenti anche reazioni ossidative. Inoltre, i formaggi congelati hanno mostrato una modifica dello stato dell'acqua da un punto di vista molecolare e mesoscopico, come conseguenza della degradazione della caseina e del cambiamento conformazionale, che ha causato la disidratazione delle proteine. La qualità del latte e la composizione del formaggio devono essere controllate con precisione al fine di limitare la presenza di enzimi proteolitici e lipolitici nel formaggio e per ottenere buoni risultati dopo un prolungato stoccaggio congelato. Inoltre, per il formaggio congelato dovrebbe essere considerato un periodo di durata di conservazione più breve rispetto al formaggio fresco.

Per quanto riguarda il formaggio P-R grattugiato, il comportamento in grattugia di diversi formaggi è risultato essere correlato alle proprietà strutturali delle diverse zone della forma; in particolare le particelle di crosta mostravano diverse proprietà di particle size e in generale erano caratterizzate da dimensioni inferiori se confrontate con particelle della parte interna del formaggio. In base a queste differenze, è stato possibile prevedere con successo le variazioni percentuali delle dimensioni delle particelle e della crosta grazie alle misurazioni nel vicino infrarosso e di Image Analysis. Queste tecniche possono essere applicate con successo dai caseifici come misurazioni interne di controllo qualità per conformarsi sia al disciplinare DOP che ai programmi di qualità interni.

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General list of abbreviations

a_w	water activity
CA	cluster analysis
CFD	computational fluid dynamics
CN	casein
C_p	specific heat capacity (J kg^{-1} $°C^{-1}$)
ES	expressible serum
FEM	finite element method
HCA	hierarchical cluster analysis
HM	high-moisture
HPLC	high performance liquid chromatography
IA	image analysis
IQF	individually quick frozen
k	thermal conductivity (W m^{-2} $°C^{-1}$).
LCA	life-cycle assessment
LM	low-moisture
LV	latent variable
NIR	near infrared
NMR	nuclear magnetic resonance
ODE	ordinally differential equation
p	pressure (Pa)
PAGE	polyacrylamide gel electrophoresis
PC	principal component
PCA	principal component analysis
PDE	partial differential equation
PLS	partial least squares
P-R	Parmigiano-Reggiano
T	temperature (°C)
t	time (s)
TPA	texture profile analysis
U	velocity vector (m s^{-1})
V	volume (m^3)
WHC	water holding capacity
δ	Kronecker delta (-)
η	dynamic viscosity (Pa s)
ρ	density (kg m^{-3})
∇	laplacian operator

1. Introduction

1.1General context: innovation and improvement in the dairy sector

Cheesemaking and cheese production is one of the oldest forms of milk preservation and processing; in particular, in Italy, cheese manufacture has a very long history, that was strongly developed at the time of the Roman Empire (Gobbetti, Neviani, & Fox, 2018). Thus, many Italian cheese products have a strong connection with technological practices that have a relatively old tradition.

Despite the long tradition, the dairy sector is continuously evolving, in order to correspond the offer to the increasing demand of high-quality products, the globalization of markets and the increasing exportation volumes of Italian cheeses, and to the compliance with regulations.

Global exportations of Italian cheeses in terms of volumes, increased from 247,008 tons of 2008, to 418,443 tons of 2018, that corresponds to a percentage increase of almost 70% in a 10-years period (CLAL, 2019). In 2018, volumes of Italian cheeses exported in extra European countries are 100,271 tons that correspond to about the 24% of the total volumes exported.

Food consumption behaviors are changing, and the food products must be able to provide greater service content; this can be achieved, for example, by reducing the frequency of purchasing products at retails by consumers, by increasing storability and/or reducing the efforts and time to prepare the food ready for consumption. As Italian dairy sector is a repository of tradition, this condition could be a powerful driver that can be used to build up new, different trends of innovation if compared to the evolution of the dairy industry in many other countries.

Facing the challenges given by the new consumers habits, both in Italy and in the world, the Italian dairy industry has the need to use scientific and technological advances to improve competitivity of Italian dairy products with products from other countries (e.g. "Italian sounding" products), but at the same time to keep the traditional identity.

In this context, there is the need to continuously promote quality of Italian cheeses and to comply with European and extra European quality schemes, but also to experiment new ways to manufacture, store and export Italian cheeses, in order to develop more efficient and sustainable supply chains that could lower the impact on greenhouse gas production of cheese manufacturing and distribution and that could further improve market share.

In order to compete with non-Italian Mozzarella, Italian Mozzarella cheese manufacturers have to increase their presence on foreign markets. In this context, the high perishability of Italian Mozzarella cheese is a strong limitation for its exportation, that can be overcome by the freezing process; however, understanding the limitations of the freezing process, the ways to improve it and how to correctly manage the frozen storage, are critical points that have to be evaluated to be able to perform a well-designed process and to obtain good quality results.

Another category of products characterized by an increasing worldwide request is represented by PDO grated hard cheeses: grated Parmigiano Reggiano and Grana Padano cheeses. To assure excellent quality characteristics of this category of products, that are also regulated by PDO standards, Italian PDO cheese manufactures need to be able to efficaciously and efficiently control the characteristics of their grated products, also in relation with the process.

To reach these goals, complex relations have to be understood; the development of chemometric and computational tools can be useful to perform this task, in particular when many process parameters and product's characteristics have to be evaluated at the same time.

1.1.1 Mozzarella cheese freezing: a method to improve product's market share and convenience

High-moisture (HM) Mozzarella cheese, is the first exported Italian cheese in terms of volumes with 98.588 tons exported in 2018 (CLAL, 2019). Italian Mozzarella cheese is a high moisture cheese, stored into a covering liquid, characterized by a short shelf life that is preferably consumed as a fresh product (Mucchetti, Pugliese, & Paciulli, 2017). For the characteristic of "freshness", Mozzarella cheese needs to fastly reach the final market of destination, as the time of transport can cover a significant part of the total shelf life; thus, in order to reach long-distance commercial markets (i.e. USA, Japan) this product is usually transported by fast means of transport (i.e. air transport). However, this kind of organization, by increasing the costs of transport and distribution of Mozzarella cheese, raises the final price of the product in the market of destination.

Dalla Riva et al., (2017), by performing a life cycle assessment study of Italian HM Mozzarella cheese, showed that energy and fuel consumption during were the main contributors in determining the post-production environmental impact of the product. In particular, transportation of Mozzarella cheese was relevant for human toxicity and photochemical oxidant formation. Authors concluded

that, among the others, an improvement of efficiency for transport activities should be reached to increase environmental sustainability, in Mozzarella cheese supply chain. On the contrary disposal of Mozzarella cheese waste (i.e. product withdrawn from the market or disposed by households at the end of shelf life) had a lower impact on the global carbon footprint of Mozzarella cheese, despite authors did not take into account the disposal at food services and restaurants due to the lack of available data. On the contrary, another study highlighted the importance of reducing product's waste along the supply over the environmental impact on Mozzarella cheese; as the production of raw milk is the major factor affecting all LCA impact categories, efforts to reduce milk or cheese loss at all stages in the supply chain have significant potential to reduce the overall impacts of cheese consumption (Kim et al., 2013).

Despite Mozzarella cheese waste did not show an important impact over the overall LC impact of Mozzarella cheese, it showed a strong impact (more than 40%) on the marine eutrophication. However, HM Mozzarella cheese disposal was estimated to be 2% in distribution and retail, and 9% in households (Dalla Riva et al., 2017; WRAP, 2014), and this percentage can be reduced by increasing storability and convenience of the products.

Moreover, Mozzarella cheese disposal is calculated in terms of waste treatment, but it should not be neglected that the wasted product, is the sum of wasted energy, materials, etc. during the previous manufacturing phases; in other terms, greenhouse gas emissions caused by production of food that gets lost or wasted are also emissions in vain (FAO, 2011), in particular by considering that dairy industry produces a multiplier effect of impacts, as a large amount of milk is transformed to make a small quantity of cheese (Falcone et al., 2017). In addition, the ethical considerations about food disposal cannot be forgotten.

In this context, in order to better preserve freshness and quality characteristics of HM Mozzarella cheese, and then to extent the shelf life period of the product, several techniques have been evaluated during the last years, such as the utilization of antimicrobial extracts (Falcone et al., 2017; Gammariello, Di Giulio, Conte, & Del Nobile, 2008), the application of bio based coatings (Angiolillo, Conte, Zambrini, & Del Nobile, 2014; Lucera et al., 2014), also in combination with modified atmosphere packaging (Conte, Gammariello, Di Giulio, Attanasio, & Del Nobile, 2009; Del Nobile, Gammariello, Conte, & Attanasio, 2009; Mastromatteo, Conte, Faccia, Del Nobile, & Zambrini, 2014), and the utilization of different covering liquid formulations (Faccia, Mastromatteo, Conte, & Del Nobile, 2012).

Among the evaluated techniques, freezing seems one of the most interesting and promising to preserve HM Mozzarella cheese characteristics for a long-term period, in particular for products characterized by relatively small dimensions (Conte et al., 2017).

Considering the relatively large impact over supply chain efficiency, patents of HM Mozzarella cheese or curd freezing have been published (Coker, Gillies, Havea, & Taylor, 2016; Zambrini & Bernardi, 2017) and nowadays, the process is performed in industrial scale also by some large companies producing Italian HM Mozzarella cheese (Granarolo Group, 2019).

However, despite the proofed industrial applicability of the process, to the date, there are not many scientific evidences of the effect of freezing and frozen storage on HM Mozzarella cheese characteristics. On the contrary, several studies were carried out to assess the applicability of freezing and frozen storage on low-moisture Mozzarella cheese characteristics (Bertola, Califano, Bevilacqua, & Zaritzky, 1996; Califano & Bevilacqua, 1999; Diefes, Rizvi, & Bartsch, 1993; Graiver, Zaritzky, & Califano, 2004; Kuo, Anderson, & Gunasekaran, 2010; Kuo & Gunasekaran, 2003, 2009; Oberg, Merrill, Brown, & Richardson, 1992; Reid & Yan, 2004; G. G. G. Ribero, Rubiolo, & Zorrilla, 2007; G. G. Ribero, Rubiolo, & Zorrilla, 2009).

According to the United States Code of Federal Regulation (Title 21, Section 133-157) the moisture content of Mozzarella cheese ranges from 2 to 60%, while for low moisture variety ranges from 45 to 52%. Storage of Mozzarella cheese into covering liquid is not provided (United States Food and Drug Administration, 2019).

Despite there was no consensus among the reported results, freezing showed some detrimental effects on cheese quality properties. In particular, it has been reported that freezing can affect cheese structure, both from a microscopic and mesoscopic scale. Ice crystal growth, that is inversely related to the freezing rate, can damage Mozzarella cheese matrix by creating voids in the casein matrix (Kuo & Gunasekaran, 2009) and can lower viscoelastic and textural properties (Graiver et al., 2004). On the contrary, casein dehydration phenomena that can be due to the modification of tertiary and quaternary casein structure, can improve hardness and consistency of the cheese (Diefes et al., 1993). Moreover, attention should be payed to the residual activity of proteolytic and lipolytic enzymes, that which activity may not be stopped also at freezing temperatures during frozen storage, or that can show a different reaction rate after thawing (Graiver et al., 2004).

Freezing of HM Mozzarella cheese can be industrially performed by the utilization of fluidized bed systems or air-blast tunnels to obtain individually quick-frozen products (IQF) by imposing high temperature gradients and convective heat transfer coefficients. On the contrary, medium sized and small dairies use air-blast freezers. Considering its high-moisture content, its relatively small dimensions and its spheroidal shape that can also be non-regular, a proper prediction of freezing times and temperature profiles should be obtained in order to improve process efficiency and to reduce energetical costs of freezing, that is one of the most energy demanding processes in food industry. Finally, manufacturers follow different behaviors facing the choice between freezing of HM Mozzarella cheese with or without the covering liquid; empirical results are often the driving force for the choice in industrial environments.

1.1.2 Particle size properties and presence of rind in grated Parmigiano-Reggiano cheese: critical properties that define product's quality and compliance

Parmigiano–Reggiano (P–R) cheese is a cooked, hard, long-ripened cheese made in Northern Italy in a restricted area around Parma, Reggio Emilia, Modena, Bologna and Mantua; P-R is one of the most famous and valuable traditional Italian cheeses and it is registered as a Protected Designation of Origin (PDO) in European Union (European Commission, 2019). According to the regulation, the PDO cheese must be ripened at least 12 months and must be produced in big cheese wheels, with a minimum cheese weight of 30 kg. Along by being one of the most famous Italian cheeses in the world, P-R is also one of the most faked Italian food products, by many "Italian sounding" products. In order to contrast the spreading of "Italian sounding" products P-R cheese products should be able to reach consumers desires and to keep excellent quality standards.

One of the most important sub-categories of P-R cheese is the grated cheese-type. Grated cheese is a particular category of the dairy market that has been subjected to a strong increase of worldwide produced and sold volumes. This is due to the high convenience of this type of product, that reach the behavior of new consumers. In the case of P-R cheese, grated cheese accounted for 5,519 tons of volumes produced, corresponding to 16% of the overall market of P-R cheese in 2018 (Parmigiano Reggiano Consortium, 2018).

While the whole wheel of P-R cheese is efficaciously identified by the marks of origin and quality impressed on the rind, the grated cheese may be more easily counterfeit both using different

cheeses, both changing some steps of the process (e.g. introducing an air- drying step that allows the use of cheeses with an inferior time of ripening, or grinding a larger part of rind).

The new mode of offer P-R cheese to consumers (e.g. small individually wrapped cubes or sticks of cheese without rind) created an excess of edible cheese rind. Dairies have to find a convenient use for this part of the cheese, so to avoid or reduce food waste. Beside the small amount of rind sold as it is, the larger part may be used as ingredient of mixtures of grated cheeses or of processed cheeses.

Considering the limitation of rind imposed by the PDO regulation of the cheese, that should be less than 18% (w/w) (European Commission, 2019), industrially, grated PDO P-R cheese can be produced by grating and mixing either the inner and the rind parts of the cheese in variable amounts.

The risk to modify the characteristics of grated cheese introducing an excessive amount of rind is easily perceivable.

The presence of excessive amounts of rind in grated cheese can be perceivable by the consumer and can have a great negative impact on the sensory characteristics (Zannoni & Hunter, 2015) that are important intrinsic quality product parameters, has they can influence market share and product appeal over consumers, also in the case of grated P-R cheese (Silvestri, Aquilani, Piccarozzi, & Ruggieri, 2019).

According to the big size of the cheese, cheese making of P-R cheese is characterized by a temperature gradient in different locations of the cheese, that can consequently cause different acidification rates (Giraffa, Rossetti, Mucchetti, Addeo, & Neviani, 1998; Pellegrino et al., 1997). Salting and ripening further improve the differences of physical and chemical properties among different locations. These phenomena can promote the presence of gradients of salt, moisture, water activity, enzymatic activities etc. that can influence the structure and modulate the rate of biochemical reactions along the different spatial locations in the cheese wheel (Malacarne et al., 2009; Sforza et al., 2012; Tosi, Sandri, Tedeschi, Malacarne, & Fossa, 2008); these gradients can be even present in cheeses with smaller size, and can promote a different texture (Alinovi, Rinaldi, & Mucchetti, 2018).

Cheese rind in ripened, hard cheeses is exposed to environmental conditions (i.e. air, water vapor pressure, light; surface microorganisms, sodium chloride) for a long ripening time, that contribute to differently modify the initial properties of the cheese matrix, if compared with the inner part of the cheese. In particular P-R cheese rind is subjected to the decrease of moisture content and water

activity, and the consequent decrease of proteolytic activities operated by indigenous proteinases (Cattaneo et al., 2008), and the increase in the degree of oxidation caused by prolonged contact with air and light (Karoui, Dufour, & De Baerdemaeker, 2007).

Along with the limitation concerning the percentage of rind, grated PDO P-R cheese must comply also with the percentage of fines: particles having diameter lower than 0.5 mm, must be less than 25%.

As in the case of cheese rind, also the presence of high amount of fine particles can negatively affect the appearance of the grated cheese and then its sensory evaluation (Zannoni & Hunter, 2013) and functional properties (e.g. melting properties). In addition, the presence of small particles can be related to rind grating, and the measure of size particles is a tool to better guarantee the quality of the grated cheese.

The improvement of the method to measure of particle size and shape can also allow to better manage some steps of the grating process.

Differences in particle size properties produced by grating processes can also promote differences and non-homogeneities in terms of product's characteristics; differences in pneumatic conveying transport of the grated particles should be expected (Deng & Bradley, 2016), with finer particles that move faster as a consequence of the weight. Moreover, differences can also arise as a consequence of sedimentation phenomena during filling/packaging operations and during transportation and storage of grated cheese bags.

1.2Statistical, chemometric and computational approaches in the dairy sector

1.2.1 Statistical and chemometric approaches

To study and evaluate the significant effect of different process conditions, especially when several factors and levels must be assessed, and when several product's quality parameters are evaluated, the accurate application of complex univariate and multivariate statistical analysis techniques has to be considered.

In dairy sciences, when the effect of several parameters in processing studies is evaluated on product's characteristics, the experimental design is often organized as a complete block design or randomized complete block design (Bello et al., 2016).

For example, in the case of in-batch processes, the experiments can be usually affected by a certain variability given by differences in process conditions, that can be still present in small amount also if the process is controlled, or by differences in treatments characteristics (e.g. cheeses to be treated by the process). The second phenomenon is usually the largest in dairy experiments involving cheese making, as batch of products that are produced in different days can be affected by a certain degree of variability given by milk composition and characteristics (Alinovi, Cordioli, et al., 2018; Schoen, 1999). On the contrary, the effect of process runs (e.g. in-batch baking or freezing) can be critical when considering industrial processes if it is not possible to consider all the factors' levels with a single run.

In order to take into account the degree of variability given by these random factors, the experiment is blocked by considering these factors; 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).

Moreover, when there are processing factors that are difficult, expensive or time-consuming to change (hard-to-change factors) between successive processing runs it is preferable to perform all the runs of a particular level combination of the hard-to-change factors (Jones & Goos, 2009). In these cases, randomization of the experimental runs (such in the case of the complete randomized block design type) is not possible and it leads to a particular type of fractional factorial design of experiment that is called split-plot (Jones & Nachtsheim, 2009; McLeod & Brewster, 2004). A split-plot design is divided into two strata or layers: an upper layer, which contains the whole plot, and a lower layer that contains the individual subplot runs (Jones & Goos, 2009). In this case the design of

experiment can be written as $2^{(n1+n2)-(k1+k2)}$, where n1, n2 and k1, k2 are the levels of each factor considered and number of added factors, respectively. It is also possible to consider more than two strata in a design, when several factors are evaluated in the same experiment: in these cases, the design of experiment is called split-split plot. This design experiment can by analyzed by a univariate polynomial model, an ANOVA model, if we are in the presence of a balanced experiment (Jones & Nachtsheim, 2009). For example, in the case of two-factor factorial design, the model can be expressed as follows:

$$Y_{ijkl} = \mu + \alpha_i + \beta_j + (\alpha \times \beta)_{ij} + \delta_i + \gamma_{ijk} \quad (1)$$

Where Y_{ijk} is the selected response variable, μ is the model intercept, α_i and β_j are the whole plot and the sub plot effect, respectively, $(\alpha_i+\beta)_{ij}$ is the interaction effect, δ_i, γ_{ijk} are the main plot and the subplot error terms, respectively.

The combined statistical strategy of the blocking design of experiment together with the fractional factorial organization (split-plot) is also be possible and is advantageous with the simultaneous presence of a hard-to-change and a nuisance factor (McLeod & Brewster, 2004).

In this case, the design of experiment (assuming again a two-factor design) can be analyzed as follows:

$$Y_{ijkl} = \mu + \alpha_i + K_j + (\alpha \times K)_{ij} + \beta_k + (\alpha \times \beta)_{ik} + \gamma_{ijk} \quad (2)$$

Where K_j represents the blocking, or nuisance factor's effect, and $(\alpha \times K)_{ij}$ is considered as the error term of the whole plot.

In order to evaluate the overall effect of different factors on several product's characteristics or to estimate and predict a product's property according to a high number of evaluations, a multivariate statistical approach may be used. Multivariate statistical techniques can be divided in supervised or unsupervised methods.

In unsupervised methods, observations have not to be labeled, but are automatically processed by the statistical algorithm in order to find a possible pattern or classification in the dataset. This kind of approach is the one used, for example, in Principal Component Analysis (PCA) and clustering techniques.

PCA is a multivariate learning technique that can be used to observe a classification pattern among samples. The principle of PCA is to reduce the large number of variables of a complex dataset from

an *m*-dimensional space into a *n*-dimensional space, with $n < m$. Usually, the space representation results to be two-dimensional (n=2), in order to have a simple and clear explanatory representation of results (Zupan, Novič, Li, & Gasteiger, 1994). PCA is based on the linear combination of the correlated measured variables to create derived variables, the principal components (PCs), that are pairwise orthogonal, non-related variables in the principal component space (Gardiner, 1997; Verdini, Zorrilla, Rubiolo, & Nakai, 2007). PCA can then be useful in the case of multicollinear datasets, where the number of *p* variables is generally larger than the number of observations, *n*. The results of a PCA is a number of PCs that resume and maximize the captured variance in the original variables. Each PC explains a different amount of variance related to the original dataset, and PCs are numbered in relation to the explained amount of variation in the original dataset, from the highest to the lowest, so that the PCs account for the highest amount of variation. PCs can be written in form of linear equations as follows:

$$PC_1 = a_{11}X_1 + a_{12}X_2 + \cdots + a_{1p}X_p \quad (3)$$

$$PC_2 = a_{21}X_1 + a_{22}X_2 + \cdots + a_{2p}X_p \quad (4)$$

$$PC_n = a_{n1}X_1 + a_{n2}X_2 + \cdots + a_{np}X_p \quad (5)$$

Where *a* represent the loading coefficient of each variable *X*, also named as eigenvectors, on the PC, and *p* is the number of original variables; the higher the loading value, the more important is the weight of the variable in the definition of the PC. Along with the eigenvalues, that represent the percentage of variation explained by each PC, the eigenvectors can be calculated by decomposing the variance-covariance matrix of PCA (Verdini & Rubiolo, 2002).

Observations can then be represented in the created PCs dimensional space. PC scores of each observation gives evidence of sample grouping in the PC space according to similarities in their characteristics; moreover, the examination of the PC loading considers the influence of the original variables in the sample arrangement.

PCA is widely applied in dairy science. PCA is applied for dimension reduction purposes in proteomic studies to find grouping of samples when several metabolites are assessed (Verdini & Rubiolo, 2002; Verdini, Zorrilla, & Rubiolo, 2004); in the case of sensory and rheological studies, to highlight relations among sensory and instrumental techniques (Brown et al., 2003; Foegeding & Drake, 2007; Medeiros et al., 2013; Truong, Daubert, Drake, & Baxter, 2002); as an explanatory statistical analysis to observe possible patterns across the dataset in spectroscopy and chemometric studies (Alinovi, Mucchetti, &

Tidona, 2019; Cevoli, Fabbri, Gori, Caboni, & Guarnieri, 2013; Cevoli, Ragni, Gori, Berardinelli, & Caboni, 2012).

Other than PCA, another kind of unsupervised multivariate analysis is Cluster Analysis (CA). CA is similar to PCA as it is a classification technique that has the scope to find clusters in the dataset, based on particular criteria that are defined as a function of the type of technique used (Sablani, Datta, Rahman, & Arun, 2006). CA is based on the calculation of the similarity or distance matrix, that is a numerical representation of similarity or differences between selected cases of the dataset. In general CA rationale is to maximize between-cluster variation and to minimize within cluster variation (Hair, Black, Babin, & Anderson, 2013). CA can be subdivided into two categories, that are based on two different principles: hierarchical CA (HCA), nonhierarchical CA. HCA can be subsequent divided in divisive or agglomerative techniques. Divisive HCA techniques start from a single cluster in the dataset, and split it into an increasing number of clusters in subsequent steps; agglomerative HCA procedure start from a number of clusters that correspond to the number of objects or observations in the dataset, that are gradually lowered in subsequent steps, according to objects' similarities.

Several HCA algorithms and clustering criteria exist and has been applied in dairy studies; most applied algorithm are the nearest neighbor with Euclidean distance criterion, the furthest neighbor approach, the Ward's method, the centroid method with squared Euclidean distance criterion, average linkage method (Pereira et al., 2016; Pripp, Shakeel-Ur-Rehman, McSweeney, & Fox, 1999; Sablani et al., 2006).

Supervised techniques are statistical approaches that are based on the relation between a dataset of variables observed and a response variable; in this category of techniques a training set is often used to create a predictive model. Most important examples of this category are Partial Least Squares (PLS) regression or discriminant analysis, Artificial Neural Networks (ANN), factorial discriminant analysis (FDA), soft independent modeling of class analogy (SIMCA).

Among the others, PLS is one of the most used supervised techniques used to build chemometric models in dairy science. PLS regression applies variables decomposition to reduce the quantity of given information by the data array, relating X-array to a Y-vector or matrix (response variable or matrix of variables). PLS algorithms elaborate multivariate data by maximizing the covariance matrix between X and Y-arrays. The result of this process is the creation of a number of Latent Variables (LVs) that are similar to PCs in the case of PCA, and are a reconstruction of the original variables and

that are highly correlated with the Y-response. PLS regression is often used in dairy science to build predictive models in the case of spectroscopic techniques (Alinovi et al., 2019; Cevoli et al., 2013) and other instrumental and sensory analyses (Candioti, Alonso, & Hynes, 2007; Truong et al., 2002).

1.2.2 Mathematical computational modelling in the dairy sector

Mathematical modelling of food processes and products is an important area of research in food science, as it is useful to better understand and investigate food modifications during processing and the impact of different treatments and process parameters. In particular, considering the continuous innovation in food processing, mathematical modelling is an important technique that can be adopted to study new processes, understand and find the best way to control critical parameters that have a significant impact over product's characteristics. Modelling of food processing can be a complex task as food products are complex systems, and can undergo several transformations during a single operation unit (Trystram, 2012).

While a minor part of mathematical problems can be solved analytically, the major part of mathematical problems has to be solved by applying numerical methods. Numerical methods are based, in the general form, on the calculation of partial differential equations (PDE) concerning the conservation of mass, momentum and energy, as follows:

$$\frac{\partial \rho}{\partial t} + \nabla \cdot (\rho U) = 0 \quad (6)$$

$$\frac{\partial \rho U}{\partial t} + \nabla \cdot (\rho U \cdot U) = \nabla \cdot (-p\delta + \eta(\nabla U + (\nabla U)^t)) \quad (7)$$

$$\frac{\partial \rho C_p T}{\partial t} - \frac{\partial p}{\partial t} + \nabla \cdot (\rho U C_p T) = \nabla \cdot (k \nabla T) \quad (8)$$

In the case of heat conduction in solid systems, mass and momentum energy can be neglected in some cases and only equation 1.8 can be considered; on the contrary, for computational fluid dynamics (CFD) studies, the fluid flow equation is coupled with the energy and mass transfer equations. Moreover, several improvements in the design complexity and accuracy can be made to study particular phenomena, by implementing models to study fluid turbulence, multiphase systems, phase change phenomena, chemical reactions (Norton, Tiwari, & Sun, 2013; Sablani et al., 2006; Xia & Sun, 2002).

To solve the system of PDE, three main numerical techniques can be adopted to develop mathematical models of food products and processes: the finite difference technique, the finite element technique and the finite volume technique. The main difference of these three methods is the spatial integration of governing equations. In order to be solved, these equations have to be expressed as ordinally differential equation (ODE) systems in discrete spatial volumes (control volumes) that are differently defined according to the three different discretization techniques. The control volume in a model is a mathematical description of the space or the geometry to be solved (Norton et al., 2013). In the case of FEM, the boundary and inner part of the domain are subdivided by lines into a finite number of discrete sized finite elements and a number of nodal points are established with the mesh (Akin, 2005).

The finite difference technique can be applied on simple shape geometries but it is characterized by difficulties to be implemented in irregular geometry studies (Yanniotis & Stoforos, 2014). To study complex geometries, where there is the need of developing an unstructured meshing, finite element and the finite volume techniques have to be used. While finite volume techniques are largely applied in the case of CFD studies, finite element methods (FEM) find applications in the case of heat transfer problems in the case of solid systems and in the case of structure analysis (Norton et al., 2013; Pham, 2006).

Once the domain of the mathematical problem is meshed by applying the chosen method and the ODE are discretized, mathematical solvers can solve the system of ODE equations for each control volume until an acceptable convergence is achieved (Xia & Sun, 2002). Mathematical solvers can be differentiated in direct and iterative solvers. The direct solvers work better than iterative solvers for simpler mathematical problems, as they will always converge to the same solution, but they will fail to reach a solution in the case of complex (Katz, 2012; Stute, Krupp, & von Lieres, 2013).

Because it is powerful to understand and model phenomena in food processing, and it is relatively cheap, fast and reliable, computational modelling has found many applications in dairy sciences. Some of the most important applications in the dairy sector are to model the kinetics of cheese drying during ripening (Castell-Palou et al., 2012), the mechanical properties of cheese (Vandenberghe et al., 2017), temperature and flow distribution into cold stores used for cheese ripening (Giametta, Fucci, Catalano, & Fianza, 2012), the heat treatment of milk and whey solutions and proteins with different heat exchangers (Chantoiseau, Plana-Fattori, Doursat, & Flick, 2012; Grijspeerdt, Hazarika,

& Vucinic, 2003; Grijspeerdt, Mortier, De Block, & Van Renterghem, 2004; Rinaldi et al., 2018), the spray-drying process of milk (Kuriakose & Anandharamakrishnan, 2010).

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2. Aim of this thesis

The aim of this PhD thesis was to improve knowledge of critical process parameters concerning freezing of HM Mozzarella cheese and the grating behavior of P-R cheese in order to improve final product's quality characteristics and market share. To do so, attention was given to the relations between products and processes.

In this context, the work was divided into two main parts, corresponding to the two cheeses evaluated, and in the case of Mozzarella cheese into 4 sub-parts:

A. To perform a literature review about the effect of freezing on dairy products (milk, cheeses, curd), in order to have a better understanding of the chemical, physical, technological, microbiological and sensory modifications involved and to formulate hypotheses about the possible mechanisms involved;
B. To study the freezing process of HM Mozzarella cheese by modelling the heat transfer during the phase change phenomenon, in order to predict the impact of process parameters, and to be able to monitor, control and improve efficiency of the process;
C. To experimentally evaluate the effects of different freezing and thawing conditions and the presence or absence of covering liquid during freezing and thawing processes on HM Mozzarella cheese characteristics, in order to find the best process conditions that have the lower impact in terms of quality decrease;
D. To evaluate the effects of prolonged frozen storage and subsequent refrigerated storage on Mozzarella cheese, over several physical, chemical and sensory characteristics, in order to evaluate quality decrease and to estimate the shelf life period of the frozen-thawed product.
E. To improve the knowledge about the grating behavior of P-R cheese in relation with the physical, chemical properties of the different parts of the cheese and to evaluate the applicability of Near Infra-red spectroscopy (NIRs) and Image Analysis (IA) coupled with multivariate techniques as industrial quality control measurement to characterize particle size properties and estimate rind's percentage of grated P-R cheese.

3. Freezing of milk, curds and cheese – a review

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3.1 Abstract

When thinking of the freezing process in dairy, products consumed in frozen state, such as ice creams and dairy ices come to mind. However, with the increasing interest to reduce food waste and improve its shelf life, to contribute to a more sustainable supply chain, and to reach new markets for dairy products, freezing processes are increasingly applied to other dairy products. Freezing is a solution to production seasonality, or to extend the market reach of high quality products with a short shelf life. This review focuses on the physical and chemical changes occurring during freezing of milk, curds and cheeses. The process of freezing is energy consuming, and needs to be optimized to maintain product quality and reduce its environmental foot print; furthermore, the processing may have to be modified as the steps leading to the freezing stage may require some changes compared to traditional, fresh products. Formulations may need to be adjusted to better control the quality of the product during the freezing and storage stages. Caution has to be taken in the intermediate stages of freezing and thawing, to minimize unwanted reactions occurring at low water activity, and as well as during storage, to avoid ice recrystallization. In freezing dairy products, attention should be given to the presence of residual proteases and lipases, as well as the modifications occurring in the colloidal–soluble equilibrium, during the cryo-concentration process. The chemical modifications occurring in these intermediate stages can have detrimental effects on rheological, appearance and taste of the final product at consumption.

Keywords: Freezing; Frozen Storage; Cheese; Curd; Dairy processing.

3.2 Introduction

Freezing is one of the most popular and common methods of food preservation. During freezing low temperatures are applied to the product to transform liquid water into ice crystals through removing the latent heat of crystallization. The product undergoes a phase transformation, from a liquid, colloidal suspension (in the case of milk and milk beverages) or a gel network (in the case of cheese), to a mixed phase containing ice crystals and a supersaturated solution. Depending on the composition of the serum, there will be a significant decrease in water mobility and mass transfer kinetics, leading to an amorphous or glassy state.

During frozen storage, the growth of microorganisms is stopped or delayed. Depending on freezing rate, microbial death or damage may also occur to the cells. The kinetics of chemical and enzymatic reactions, such as proteolysis and oxidation, as well as physical changes, such as mass transfers (i.e. recrystallization or phase separations) are minimized (Delgado and Sun 2001; Sun 2016), as a consequence of low water activity (a_w) and decreased molecular mobility. In general, the lower the freezing temperatures are, the slower the kinetics of the deteriorative reactions (Verdini and Rubiolo 2002).

However, freezing, thawing and storage need to be well controlled, as they can result in quality losses (Fu and Labuza 1997). This is caused by several factors which affect the reaction kinetics, such as freeze-concentration effects, catalytic effect of ice crystals, a greater mobility of protons in ice than in water, a_w. Moreover, structural, textural, and rheological modifications occur as well as changes in sensory properties, during storage or after re-equilibration at higher temperatures.

A significant amount of research has been carried out to study how to control ice-crystal size and growth. The formation of the ice crystals, and the control of nucleation and growth have been extensively reviewed (Cook and Hartel 2010), as it is critical to the quality of frozen dairy products such as ice cream and frozen desserts. However, the changes occurring to other dairy products, such as milk, curd and various cheeses, during freezing and storage, are less understood. This area is of growing interest, as freezing improves the presence of high-quality foods in markets with no access to fresh specialty dairy products (Tejada et al. 2002; Bertola et al. 1996a). Freezing also allows for innovation of ingredients and semi-finished products to be further processed at destination (Picon et al. 2013; Vélez et al. 2015).

Considering the growing global interest for more sustainable supply chains, despite the high energy consumption of the process (Baldwin 2009), freezing may be considered a good solution in a total lifecycle analysis, when adoption of the process results in food waste reduction. Furthermore, freezing increases accessibility of certain products to new markets. Because of the greater storability and convenience (Pollack 2001), some frozen foods have shown to have a lower impact on the carbon footprint if compared with fresh, refrigerated products consumed in households, due to the decrease in food waste (Martindale 2014). Moreover, frozen foods are a solution to seasonality challenges (Anaya Stucchi and Pollitt 2019). Freezing can be a solution to the increased global demand for highly perishable traditional cheeses (e.g. high moisture controlled origin cheese), by reducing the costs of transport (maritime vs air) (Estrada-Flores 2016) and in this case also, decrease waste, for example, as a result of expired product during transport delays. For example, overseas export of Mozzarella cheese manufactured in Italy increased by 16.4% from 2016 to 2017, with Japan, South Korea and USA that imported more than 30% of the total amount of exported product (CLAL 2020).

As the process of freezing is energy-consuming, the correct prediction of cooling and freezing times is important to achieve better energy efficiencies, as well as to achieve the optimal quality parameters of the final product. Thanks to technology improvements and the study of processes by computational methods (Delgado and Sun 2001), new and more efficient processes have been developed (Cheng et al. 2017; J. Evans 2016), increasing both the sustainability and quality of frozen foods (Hall and Howe 2012). Moreover, optimization of supply chain (batch sizes, transportation temperatures and storage times) can further contribute to lower the impact of frozen food on the environment (Zanoni and Zavanella 2012).

Freezing process conditions deeply influence the final quality of the product, as the growth of ice crystals and formation of phase separated systems may result in significant damages to the matrix. Ice crystal growth (due for example, to Ostwald ripening), dehydration, residual activity of proteases and lipases can cause these changes; for example, during freezing, bulk water tends to freeze faster than entrapped water by biopolymers (proteins, polysaccharides). This can generate concentration gradients that result in the acceleration of reactions, protein aggregation or unwanted micro-phase separations (Fennema, Powrie, and Marth 1973). These phenomena can be detrimental to the quality of products if the freezing–thawing conditions are not well controlled, and in some cases, formulations are modified to take into consideration the changes in the unfrozen phase (Diefes, Rizvi, and Bartsch 1993; James, Purnell, and James 2015). The time in this phase transition zone, at

temperatures around -1°C to -6/-8°C (Diefes, Rizvi, and Bartsch 1993; Fikiin 2008), must also be minimized to optimize ice crystals nucleation at the expenses of their growth (Kiani and Sun 2011).

Storage temperatures also play a crucial role in maintaining quality as a lower storage temperature corresponds to a lower amount of unfrozen water available for chemical, enzymatic reactions and growth of ice crystals. In other words, the properties of the components which were preserved by applying a fast-freezing process can be lost under deteriorative storage conditions (Alvarenga et al. 2013).

For cheeses, the preservation of quality by freezing will depend on the matrix. High moisture cheeses may over-ripen very fast during refrigerated storage, shortening shelf life; in these cases, freezing can be a help to preserve cheese characteristics. However, the methods of freezing must be optimized for these products (Alvarenga et al. 2013).

In sum, freezing needs to be a well-designed process, supply chain controlled with optimized storage conditions so that no major changes on product quality will be observed during shelf life. The continuous process of freezing for ice cream type products has been optimized over the years, and it is well understood (Cook and Hartel 2010). It involves the use of scrape heat exchangers which agitate, whip, destabilize the fat and scrape the ice crystals from the surface of the barrels. This process controls the nucleation process, and after this stage, the product is further cooled to harden, with an increase in the ice crystal sizes (Cook and Hartel 2010). In this review, we will focus on describing the effect of freezing and its possible application in milk, cheese and curd products.

3.3 Microbial ecology and quality

As a consequence of the increase of solutes concentration, the subzero temperatures and the very limited molecular mobility, the microbial growth in frozen dairy matrices is generally halted. Moreover, both the freezing process and the storage at subzero temperatures may increase the mortality of microorganisms, because of the mechanical damage caused by the intracellular and extracellular ice crystal formation to the microbial membranes, to dehydration of the cells caused by water pressure differences and the presence of osmotic gradients (El-Kest and Marth 1992; Smith et al. 2011).

Coliforms show high mortality during freezing (Katsiari, Voutsinas, and Kondyli 2002; Voutsinas et al. 1995a; Voutsinas et al. 1995b) while micrococci, staphylococci and streptococci are more resistant to freezing and frozen storage. Generally, gram-negative are more sensitive to freezing than the gram-positive bacteria, cultures in the log-phase more than in the stationary phase, and vegetative cells more than bacterial spores (Speck and Ray 1977). Wendorff (2001) determined that higher storage temperatures (-15°C vs -27°C) lead to a higher microbial mortality because of the damage caused by larger ice crystals.

Concerning pathogens, Smith et al. (2011) observed that freezing in the case of sheep milk may reduce the count of *Staphylococcus aureus*, *Mannheimia haemolytica*, and *Escherichia coli*, even in the presence of cryoprotectants such as glycerol. (Papageorgiou, Bori, and Mantis 1997) observed frozen and stored ewe's milk at -18°C and -38°C for 7.5 months inoculated with both *Listeria monocytogenes* strain Scott A and California. The two strains showed a survival rate of 95% and 40-50% of the initial inoculum, respectively. The greater stability of Scott A strain compared to other strains in milk confirmed other reports (Gianfranceschi and Aureli 1996). Milk components can act as cryoprotectants. The addition of fat, lactose and caseins to phosphate buffer significantly reduces death and injuries during frozen storage at -18°C of *L. monocytogenes* strains Scott A, V7 and California, with caseins being the most protective (El-Kest and Marth 1991a; El-Kest and Marth 1991b). Similarly, only one log reduction has been reported for Staphylococcus aureus in whole milk after several months of frozen storage (B. M. Lund 2000). Freezing and frozen storage of cheese curds can increase the mortality of microbial strains of particular importance (e.g. *L. monocytogenes* strains), compared to milk freezing. *L. monocytogenes* strains Scott A and California are less freezing tolerant in cheese curd, because of the lower pH compared to milk. There are differences in the distribution within the cheese; a higher survival rate was observed in the outer part of the cheese

curd than in the center, because of the difference in the size of the ice crystals, with the smaller ice crystals forming on the surface compared to the center, due to higher freezing rates on the cheese curd surface (Papageorgiou, Bori, and Mantis 1997). Higher freezing rates are generally associated to a lower damage inflicted to microbial cells, because of the formation of a larger number of smaller nuclei, and limited ice crystal growth (Wang et al. 2019). Moreover, also the thawing temperatures and conditions and the storage after thawing are important in defining the microbial viability in the product, as during thawing psychrotolerant and psychrophilic are able to grow; by thawing 1-month frozen stored sheep milk, Tribst, Falcade, and de Oliveira (2019) observed that higher thawing temperatures (25°C vs 7°C), volumes of packaged product (5 L vs 1 L) and the presence of refrigerated storage post-thawing, caused an slight increase (<1.5 log) in the total and psychrotolerant bacterial count. Moreover, during thawing changes in electrolyte concentrations or water recrystallization during thawing can also lower microbial viability (Fernandez Murga, De Ruiz Holgado, and De Valdez 1998).

Despite a reduction in microbial counts is generally expected with freezing and storage at subfreezing temperatures, these processes and storage conditions are not a guarantee for microbiological safety (Katsiari, Voutsinas, and Kondyli 2002), especially when a long thawing time is required (Tribst, Falcade, and de Oliveira 2019). Also, freezing/thawing processes may cause sublethal damages to pathogen cells (Speck and Ray 1977), that can result in the presence of reversibly injured cells that can be able to recover in favorable conditions (Fernandez Murga, De Ruiz Holgado, and De Valdez 1998). Sierra et al. (2009) observed only a slight reduction in total microbial count (about 1 log) of frozen goat's milk stored for 24 h if compared to refrigerated milk.

The viability of LAB cultures and probiotics as a function of freezing and frozen storage has been largely studied both for the industrial production of frozen or freeze-dried starters or for the utilization in frozen yogurts and ice creams. Use of rotary freezers for quickly freezing of LAB cultures in form of small individual spheres followed by packaging is a common practice of many starter manufacturers. LAB freezing-resistance has been shown to be specie and strain specific (Fernandez Murga, De Ruiz Holgado, and De Valdez 1998). A high probiotics and LAB viability can be guaranteed by the utilization of cryoprotectants or by the adoption of microencapsulation techniques (A. G. Cruz et al. 2009; Wang et al. 2019). In particular, a mixture of trehalose, sucrose, glycerol and skimmed milk demonstrated to enhance significantly the survival rate of *Lactobacillus plantarum*, *Lactobacillus casei* at levels higher than 90% (Wang et al. 2019). Also the production of exopolysaccharides from

LAB species has been suggested to enhance cryotolerance during freezing processes or frozen storage (Monnet, Béal, and Corrieu 2003; O'Brien et al. 2016).

Freezing can also be an important factor conditioning cheese ripening. This may be of particular importance in the case of semi ripened cheese, transported frozen, which may develop different ripening characteristics once thawed, compared to the original one. This is also very important when the product is further processed. When frozen-stored curds were mixed with fresh ones, little counts of lactic acid bacteria (LAB), gram negative, mesophilic, coliforms and staphylococci bacterial counts were observed (Campos et al. 2011; Alonso et al. 2011; Picon, Gaya, et al. 2010). However, differences were found in the *Lactobacillus* population and in particular of *Lactobacillus plantarum* during ripening. Because of the complex set of peptidases that contribute to the formation of volatile compounds and flavors, the presence of a modified microbial flora throughout the ripening will promote differences in flavor and taste of the final cheese. Moreover, a higher degree of proteolysis, aminopeptidase and esterase activity is found in frozen curd, and in the cheese made with frozen stored curd, due to the presence of enzymes leaked during membranes rupture, and not inactivated by freezing (Picon, Alonso, et al. 2010). This may lead to a less balanced flavor intensity and bitterness in ripened cheese (Alonso et al. 2013).

3.4 The freezing process

In physical and chemical terms, freezing is a phase change process, which has been extensively reviewed in literature (Alvarenga et al. 2013; Zhao and Takhar 2017). As the temperature is lowered below the freezing point of the system, liquid water starts to nucleate, and ice crystals form (Figure 1). If the temperature during freezing is sufficiently low, transitions from a liquid to a rubbery state of the matrix, to a vitreous, glassy state occur (Zhao and Takhar 2017). This second phase transition is related to the solidification of water that induces freezing-concentration of salts, sugars and organic acids that significantly increase viscosity of the unfrozen matrix, leading to arrested molecular motion.

Several processes have been utilized to freeze cheese and dairy products such as conventional air-still freezing (Simov and Ivanov 2005), air-blast freezing (Diefes, Rizvi, and Bartsch 1993), spiral or tunnel freezing, plate freezing, deep immersion freezing in brine solutions (Ribero, Rubiolo, and Zorrilla 2007; Ribero, Rubiolo, and Zorrilla 2009) or in cryogenic liquids (Bertola et al. 1996a).

Air-still freezing is the most widely practiced freezing process, at all scales, but very challenging to optimize, as time-to-freeze can be very long, with variations between sizes and geometries of the product, differences depending on the location and the type of packaging set up, all parameters with an obvious effect on the final quality of the product.

In cheese, for example, temperature curves can be different depending on the size of the product, and the cooling is faster on the surface than in the central part of the cheese, especially for large size products; unfrozen water would be more abundant in the center than at the edges, and the water would diffuse in the opposite direction from freezing, from the center to the edges, resulting in overall drying of the product (Alinovi and Mucchetti, forthcoming; Norwig 1984). Moisture, solutes and temperature gradients can affect the biochemical changes occurring to the product and their kinetics: protein dehydration in cheese matrix is a consequence of water migration phenomena that take place at a microscale level (Bunker 2016; Reid and Yan 2004) (Figure 2); enzymes distribution and subsequent activity can be conditioned by water migration, and can contribute to the softening of fresh cheeses when proteases are involved (Alinovi, Rinaldi, and Mucchetti 2018); microbial death and cellular lysis can be also affected (Papageorgiou, Bori, and Mantis 1997).

To avoid surface dehydration, as glaze is not generally used for dairy products like in the case of other food freezing processes (Popelka et al. 2012), packaging materials that provide barrier to water

evaporation can be used as skin. During the freezing process of unpackaged products or during long-time frozen storage, the dehydration may cause skin burn defects, and with oxygen contact, promote oxidation reactions. This is usually higher in air-blast freezing processes (Norwig 1984). Immersion freezing into brine solutions has also been suggested as an option, but this can have also some problems related to brine concentration changes caused by mass transfer phenomena, in high viscous fluids (Fikiin 2008). A recent evolution of this method is the hydrofluidisation freezing, that combines the use of immersion brines with the application of nozzles to obtain high turbulence flow during the process (Fikiin 2008).

Air-blast freezing and plate freezing are batch or semi-continuous processes which also may result in significant thermal gradients (Campos et al. 2011). On the contrary, continuous freezers suited for smaller products, of defined size may result in very short freezing times. Tunnel freezers or spiral freezers represent a continuous application of air-blast freezing and can be optimized for different kind of products with different sizes, by modulating the dwell speed (George 1997). To maintain the product unit separated, fluidized bed systems or immersion systems can be used to ensure that each unit is individually frozen and that the freezing time is minimized. In these systems, the high heat transfer coefficient of the process is obtained by using liquid nitrogen, liquid nitrogen vapors or low-temperature air flowed at high velocity (2 – 6 m/s) as freezing medium on the product passing through an insulated tunnel or in an immersion vessel. The application of high air flow rates keeps the product in motion thus avoiding the formation of clumps and improving the heat transfer during the process.

Fluidized bed freezing technology, widely applied in foods, is gaining interest also in dairy, to obtain Individual Quick-Frozen (IQF) Mozzarella cheese and other small size high moisture dairy products, including shredded cheese. The main limitation concerning fluidized bed freezing is the dimensions and weight of the product, that has to be small enough to become suspended by the air flow (George 1997).

The cost of cryogenic-IQF is higher due to the use of cryogenic liquids (Fikiin 2008), but the product quality is superior to conventional methods (Khadatkar, Kumar, and Pattanayak 2004). IQF techniques are commonly used to freeze whole fruits of small, uniform size and not prone to damage as a consequence of the high mixing velocities (A. Chaves and Zaritzky 2018).

Direct immersion in cryogenic fluids or application of fluidized beds can cause cracking and shattering of the surface of the products (Reid and Yan 2004), precooling is necessary to prevent such defects. A possible limitation to the diffusion and applicability of cryogenic fluids is given by the high cost of production, as the nitrogen production process is energy intensive (Pearson 2008) but can be a solution for high moisture, fresh products such as HM Mozzarella cheese and curd to better preserve the structure and juiciness (Zambrini and Bernardi 2017).

Freezing processes have been tested to optimize energy use and limit undesirable mass and moisture gradients in the product and to increase the freezing rate; for example systems could be combined to apply a cryogenic pre-stage to freeze the outer layer and a mechanical final stage to freeze the core (George 1997); Magnetically and electrically disturbed freezing, microwave assisted freezing, high-pressure freezing, ultrasonic freezing, have also been suggested, for example, to promote supercooling or to reduce freezing times (Cheng et al. 2017; Fikiin 2008; James, Purnell, and James 2015; Johnston 2000). Magnetically, electrically disturbed freezing and microwave assisted freezing techniques rely on the ability of magnetic or electric fields and microwaves to modify the electric dipole moment of water and its distribution into the product; this phenomenon cause the suppression of ice formation and enhance the supercooling effect (Cheng et al. 2017). Ultrasonic freezing involves the formation of cavitation bubbles, which favor the formation of small ice nuclei and the fragmentation of big ice crystals (James, Purnell, and James 2015); moreover, as this technique combines immersion freezing with high-intensity ultrasound (usually 20– 100 kHz), it has been reported to increase the freezing rates and the convective heat flow coefficient, and to inhibit the residual activity of enzymes in a large number of applications in food sciences (Cheng et al. 2017; Kiani et al. 2011), while in the dairy sector no applications can be found to the date. Concerning high-pressure freezing caused the formation of a plasticized texture in Mozzarella and Cheddar cheeses, as products were more readily deformed and had a lower brittleness than fresh cheeses (Johnston 2000); these phenomena can be related to the effect that high pressures exhibit on proteins secondary, tertiary and/or quaternary structure and that can have a reflection on proteins' functionality (Huppertz, Fox, and Kelly 2004).

In the case of milk or concentrated milk, freezing may be applied after packaging in sealed bags to reduce as much as possible to minimize oxidation; several freezing techniques can be used, such as tunnel, spiral freezers or air-blast freezers. Batch processes, such as multiplate and plate freezing, can also be applied to obtain frozen blocks of various dimensions (Antifantakis et al. 1980); however,

volumes should be reduced as much as possible (e.g. volumes smaller than 1-5 L with 2 cm thickness) in order to decrease freezing times and ice crystal growth (Antifantakis et al. 1980; Haenlein and Wendorff 2006; Tribst, Falcade, and de Oliveira 2019). For example, a tray-multiplate freezing system that can produce small frozen milk blocks of sufficiently homogeneous characteristics and high stability has also been developed (Zevlakis 2004); this system combines a supercooling phase of the liquid to produce small crystal size and a fast heating of the solid layer between the tray surface and the frozen product to allow the detachment of the frozen segments. For continuous freezing of fluid or concentrated milk, drum contact freezers equipped with scraped blades and twin-screw extrusion device have been suggested to obtain thin flakes of frozen product prior to packaging (Addeo et al. 1992; Groux, Fayard, and Jimenez-Laguna 2001; Pirisi et al. 1995). These processes minimize freezing times, and control ice crystals dimensions. In particular, a twin-screw extrusion device can minimize freezing times due to the supercooling caused by the increase in pressure in the extruder, with a rapid and homogeneous freezing as the pressure of the low-temperature product drops down (Groux, Fayard, and Jimenez-Laguna 2001).

3.5 Physical and chemical changes of milk

The effect of freezing milk has been studied for decades (Pazzola et al. 2013; Muir 1984). For non-bovine species (sheep, goat, buffalo), seasonality issues caused by reproductive performances and/or the lack of pastures during some periods of the year have been mitigated using freezing of bulk milk (Pazzola et al. 2013). The freezing process has found its application for milk intended for further processing (e.g. fresh cheese) because of the value in having production of cheese all year long. Freezing can be advantageous for dairies, to better control the fluctuations in milk supply to be able to produce cheese more regularly, reducing the impact of fixed expenses and increasing the market availability of the cheese (Tribst et al. 2020; Tribst et al. 2019).

Milk can be frozen as is or after concentration process, such as ultrafiltration (UF), reverse osmosis or under vacuum evaporation, to reduce freezing and storage costs, as the freezing time, energy costs, storage and transportation cost would be reduced (Haenlein and Wendorff 2006). It has been suggested that, milk should be skimmed, as freezing can lead to fat globules rupture, increasing the rate of lipolysis and fat oxidation during frozen storage (Antifantakis et al. 1980; Voutsinas et al. 1995b) with consequences to the processability of this milk into fresh cheeses at destination; moreover, fat globule breakage would result in destabilization of emulsion, coalescence of globules after thawing and creaming phenomena favored by fatty acid crystallization and the activity of agglutinins (Tribst et al. 2020; Tribst et al. 2019).

Enzymatic and chemical kinetics are directly proportional to the storage temperature, and changes in composition (Needs 1992; Wendorff 2001). Storage of frozen milk can result in off-flavors, due to reactions such as oxidations, lipolysis and proteolysis. Lipid oxidation has to be controlled. In sheep's milk, if not controlled, the peroxides number and the free fatty acids content can increase by 7 to 90 times and 2.5 times, respectively, after 5 months of frozen storage at -20/-30°C (Antifantakis et al. 1980). The presence of oxygen, light and metals, especially copper and iron, that can be found in traces (Coni, Bocca, and Caroli 1999), promote the rate of oxidation. Oxidation can be reduced using oxygen impermeable (Voutsinas et al. 1995b) and light barrier packaging materials (e.g. polyethylene-aluminum foils) and by the application of thermal treatment prior to freezing, to cause inactivation of indigenous lipase enzymes (Katsiari, Voutsinas, and Kondyli 2002). It has been shown that frozen storage induces a significant deactivation of lipase enzymes after 6 months at -12°C, -20°C and -27°C, as the residual activity decreases to 2%, 11% and 24% of the initial activity values, respectively (Needs 1992).

As a consequence of lipid oxidation, the lipophilic vitamins content, also decreases during frozen storage. While short-term frozen storage does not show a significant reduction, α-tocopherol content of UHT treated milk is significantly lowered by long-term frozen storage at -20°C for 6-8 months (10-21% losses), suggesting that oxidation reactions still occurs after UHT processing (Vidal-Valverde, Ruiz, and Medrano 1993). Non enzymatic oxidative reactions, such as Maillard reactions could also create many precursors for oxidative reactions causing crosslinked amino acid products, with higher incidence in lactose free UHT milk (Jansson et al. 2014).

The residual activity of proteases also needs to be considered, as both exogenous and indigenous proteases such as cathepsin D and plasmin can cause significant quality challenges. Plasmin is an indigenous protease that can hydrolyze Lys-X peptide bonds of β-casein and is mainly active in the hydrolysis of β-casein, despite cleavage sites can also be found in α_{s1}- and α_{s2}-caseins (Kelly and McSweeney 2003). It has been shown that plasmin is active at temperatures < 5°C, and an increase in solubilization of the caseins from the micellar phase can facilitate the hydrolysis (Ismail and Nielsen 2010).

Residual microbial enzymes also have a serious impact on the physical stability and overall quality of frozen dairy products, and in milk, it is important to control the microbiological quality and the storage time in the raw bulk tanks (Portmann 1970). High microbial counts of psychrotrophic bacteria before processing will result in higher concentrations of exogenous proteases and lipases, some of them also heat resistant (Chavan et al. 2011; Tribst et al. 2019). Lipolytic and proteolytic reactions will continue to occur during freezing, and the freeze-induced concentration of solutes will increase reactions kinetics, before the supersaturated phase reaches a vitreous state.

In addition to chemical and enzymatic reactions such as oxidation and hydrolysis, during freezing a number of physical changes occur to the milk matrix, as illustrated in Figure 3. As the freezable water progressively transforms into ice crystals, the unfrozen solution becomes more and more concentrated, suppressing the phase transition temperature. Freezing can improve the opportunity of enzymatic reactions as macromolecules are forced closer together as a result of the increased concentration of the unfrozen phase; this will also increase the concentration of enzymes inhibitors (Ashie, Simpson, and Smith 1996). The formation of ice crystals (and fat crystals) and their growth will cause physical damage to the milk fat globule membrane which promote clumping and ultimately partial coalescence (Tribst et al. 2020; Zhang et al. 2006). The application of homogenization process can reduce the occurrence of this phenomenon if applied before freezing or it can be used after

thawing in order decrease the effects of emulsion destabilization (Tribst et al. 2020); however if the milk is used for cheesemaking, this practice may not be desired.

The increase in concentration in the unfrozen phase promotes also a change in the colloidal properties of the protein suspension. In normal milk, the casein micelles occupy about 10% of the volume and their average distance is in the same order of magnitude of their diameter. During freezing, the volume fraction of the casein micelles continues to increase, causing an exponential growth of the relative viscosity, and an increase of the colloidal interactions (Figure 3) (Corredig et al. 2019). Also, casein micelles may change their structure during frozen storage. It has been hypothesized that as water migrates into ice crystals, protein hydrophobic portions become exposed to the solvent, generating protein-protein interactions via hydrophobic and ionic forces and resulting in protein aggregates (Xiong 1997). Supersaturation of the unfrozen phase modifies the colloidal state and hydration of casein, resulting in salting out (Doan and Warren 1947), unfolding of the tertiary and quaternary protein structure and a change in the micellar voluminosity. The presence of micellar aggregates was shown by electron microscopy (Saito, Niki, and Hashimoto 1963). It has been previously reported that in frozen milk there is sedimentation and precipitation of the insoluble proteins, generating casein complexes (Koschak et al. 1981), a decrease of functional and technological properties and a chalky mouthfeel (Nickerson 1964). Caseins precipitation may also be related to a change in ionic equilibria; as a consequence of the increasing ionic strength of the medium during freezing, due to freeze-concentration phenomena, it is possible to assist in the precipitation of soluble calcium, a change in the buffering capacity and a decrease in pH of the medium (Kljajevic et al. 2016; Tribst et al. 2019; Van Den Berg 1961; Tribst et al. 2020). The aggregation has also been attributed to the formation of calcium phosphate bridges with the caseins (Wells and Leeder 1963), and confirmed by molecular exclusion chromatography (Chen and Yamauchi 1971). A decrease in concentration of soluble calcium and inorganic phosphate has been measured after 7 months frozen storage at -20°C, and attributed to the formation of insoluble salts of calcium citrate and phosphate and the formation of calcium-phosphate-casein complexes that cause the precipitation of proteins (Chen and Yamauchi 1969a; Yamauchi and Yoneda 1977). Accordingly, Tribst et al. (2020) hypothesized that the phenomena related to sheep's milk destabilization following freezing and thawing are due to the precipitation of soluble calcium in colloidal form; changes in mineral equilibria may cause a conformational change in casein micelles and an increase in hydrophobic interactions. This would result in a reduction of casein-fat

interactions, and in the possible formation of casein complexes, resulting in sedimentation and coalescence phenomena (Tribst et al. 2020; Tribst et al. 2019).

A higher stability has to be reported for goat's and sheep's milk after prolonged frozen storage, possibly due to a higher buffering capacity compared to bovine milk (Katsiari, Voutsinas, and Kondyli 2002; Tribst, Falcade, and de Oliveira 2019; Voutsinas et al. 1995b; Voutsinas et al. 1995a; Wendorff 2001). This higher stability resulted in products such as yogurt and cheese characterized by similar physical, sensory, microbiological and chemical characteristics to the ones obtained with fresh, refrigerated-stored sheep's milk (Katsiari, Voutsinas, and Kondyli 2002; Zhang et al. 2006). Moreover, also slow rate of thawing or a resting period in refrigerated conditions after thawing, can improve stability of milk by giving the time to resolubilize colloidal minerals before further processing (De La Fuente, Requena, and Juárez 1997; Tribst et al. 2019; Tribst et al. 2018).

In cow's milk, the supernatant fraction seems to have a higher amount of β-casein (Nakanishi and Takatoshi 1965) and κ-casein after long frozen storage time (180 days) (Chen and Yamauchi 1969b) as the increase in ionic strength during freezing that can lower the binding of calcium ions to β-casein (Kljajevic et al. 2016). It is obvious to say that, a high degree of caseins destabilization can deeply influence the functional properties of the milk, affecting its cheese making ability and consequently the final characteristics of the cheese.

It is important to point out that much work published in the past did not specify processing history conditions, nor used advanced physical tools that may be able to better elucidate the structure formed during freezing. Furthermore, the literature is lacking detailed proteomic data to be able to give a complete understanding and distinguish between colloidal instability, ionic bridging and proteolysis. Nonetheless, the ionic environment, and calcium specifically, plays and important role, because, in the absence of calcium or in the presence of chelating agents, the destabilization is reduced (Wells and Leeder 1963; El-Negoumy and Boyd 1965). Under these conditions the colloidal structure of the casein micelles is also significantly affected.

As a consequence of freezing, the destabilization phenomena, cheesemaking and product final quality can be affected; while the data for bovine milk are scarce, literature reports are available for other milk species. For example, it has been shown that there is a significant decrease of sheep milk renneting characteristics (lower curd firmness, higher renneting time), after freezing for 6 months (Pazzola et al. 2013). This may be caused by increased proteolysis. Other authors also showed

changes in the functional properties, such as a lower retention of fat in the sheep's milk curd (Lin, Hsieh, and Su 1994), and a lower gel firmness and water holding capacity (WHC) in sheep's milk yogurt (Tribst et al. 2018; Voutsinas et al. 1996a; Wendorff 2001), changes in the rennet-induced gelation of caprine, ovine and buffalo milk (Addeo et al. 1992; De La Fuente, Requena, and Juárez 1997; Kljajevic et al. 2016). In some cases, freezing may weak the electrostatic interaction among casein micelles, resulting in less structured protein matrix, a lower gel contraction and less whey expulsion (Tribst et al. 2018).

Freezing may cause a significant change in cheese actual yield. This has been shown for cheese made with frozen sheep milk (Zhang et al. 2006). The change in yield was attributed to the reduced WHC of the caseins, but also fat recovery, which can be lowered by a weaker casein matrix that can affect coagulum structure (Pazzola et al. 2013). A lower water content referred to a non-fat basis was also measured in the case of white brined cheese manufactured using frozen stored goat's milk (Kljajevic et al. 2017). In these studies, proteolysis was not tested. On the contrary, Pirisi et al. (1995) showed an increase of dry matter recovery and a higher cheese yield when sheep frozen milk has been used.

Lactose crystallization during freezing can also drive to instability. During storage, the β-lactose form is converted into the less soluble α-lactose monohydrate form (Desai and Nickerson 1964). A high degree of destabilization has been reported when 1/3 of lactose is in the α-form (Muir 1984). As lactose crystals are removed from solution, the freezing point increases, causing more nucleation and growth of the ice crystals and proteins destabilization by salting-out (Morr 1975).

Membrane filtration processes such as ultrafiltration (UF) and reverse osmosis (RO) or ultrafiltration can be used to remove ionic form of calcium, monovalent salts, lactose and/or water, respectively (Haenlein and Wendorff 2006; Mucchetti et al. 2000). The application of UF with concentration factors ranging between 1-3 times show an improvement of milk stability over storage time, that was found to be at least 3 times longer (Koschak et al. 1981). However, higher degrees of UF (4-5 times) that were able to remove high degree of soluble salts, in particular calcium (>20%), and lactose (>70%), were not able to prolong shelf life, resulting even in its reduction, as the equilibrium of colloidal calcium phosphate to soluble phosphorous is affected (Lonergan, Fennema, and Amundson 1981; Koschak et al. 1981). Also RO demonstrated to be a good concentration technique that can be applied before milk's freezing; physical stability was excellent even after 6 months frozen storage and no differences in lipolysis or fat oxidation were observed between the control milk and the frozen RO concentrates (Voutsinas et al. 1996b). Apart from RO and UF, also dialysis processes and ion

depletion, may lead to an improvement of freezing stability (Lonergan, Fennema, and Amundson 1982).

Any ingredient that is able to decrease the rate of lactose crystallization would improve stability in frozen milk. For example, other added soluble carbohydrates (sucrose, glycerol, etc.) may act as cryo-protectants, delaying lactose crystallization, by increasing the viscosity of unfrozen solution (Desai, Nickerson, and Jennings 1961; Minson, Fennema, and Amundson 1981). Lactose hydrolysis has also been suggested to improve stability, as the monosaccharides glucose and galactose are characterized by a high solubility and they have no tendency to crystallize; a linear decrease of lactose concentration showed to improve shelf life exponentially (Tumerman, Fram, and Cornely 1954). However, care needs to be taken for non-enzymatic oxidative Maillard reactions (Jansson et al. 2014).

Conditions that may involve supercooling, improve nucleation and minimize crystal growth, or that can produce a vitreous matrix with low molecular mobility, will be beneficial. Rapid freezing and cold storage at temperatures lower than -20°C, can slow lactose crystallization; instead, storage temperature ranging between -2°C and -12°C showed detrimental effects on cow milk properties such as viscosity and solubility index (Koschak et al. 1981), as these conditions create a high molecular mobility of the unfrozen phase result in a lower stability of the milk system during frozen storage.

3.6 Butter Freezing

Butter is a water-in-oil emulsion composed of more than 80% milk fat, and a water phase, that is dispersed into the fat matrix as small spherical or oval droplets (Nahid et al. 2008). Butter is commonly frozen and stored in bulk before further processing. In this case, freezing can be applied to control the product peaks in terms of volumes that generally occur during winter and to respond adequately to consumer and market demand. The freezing process of butter can be advantageous also because of the low economical and energy-demand costs that are a consequence of the low specific heat and latent heat of fusion of the product (Persson and Londahl 1993). In fact, despite butter can have a relatively long refrigerated shelf life (6-12 months), frozen storage has demonstrated to preserve better its aroma characteristics and the occurrence of off-flavors (Lozano et al. 2007).

As the major component is milk fat, butter frozen storage is mainly limited by lipolysis and oxidation; these reactions result in modification of color, structure and sensory characteristics (rancidity and other off-flavors) (Krause et al. 2008). Moreover, textural and rheological changes during freezing and frozen storage caused by a modification of the fat crystal structure into a more rigid one can also be observed (De Man and Wood 1958); in fact, the rate of cooling and temperature fluctuations are crucial in defining the presence and relative abundance of sub-α, α, β', β crystal forms of milk fat (Lopez et al. 2002; Ronholt et al. 2012) and the microstructure of the crystalline network. Higher cooling rates promote a denser crystalline network composed by smaller crystals and the formation of metastable polymorphic forms such as sub-α and α form (Wiking et al. 2009).

Main factors affect the quality of butter during freezing, namely, the packaging or wrapping material, which controls oxygen and light contact with the product surface, thus regulating the rate of oxidation (D. B. Emmons et al. 1986; Lozano et al. 2007); the presence of metals in the contact materials that induce oxidation (Krause et al. 2008); water droplets distribution and size, promoting destabilization and microorganism growth after freezing/thawing; the freezing rate, affected by the size of the product (Krause et al. 2008). Storage in 25 kg blocks seem to be improving the stability of butter (Krause et al. 2008); furthermore, frozen butter should be packaged into aluminum foils rather than into parchment paper, in order to reduce oxidation and lipolysis caused by light (Lozano et al. 2007).

3.7 Freezing of Mozzarella and Pizza cheese

Mozzarella cheese is a pasta filata type cheese; during its production the curd undergoes a stretching process which gives the cheese unique texture and melting properties. During this process, the curd is continuously kneaded and stretched at high temperature and the result is the formation of a smooth, fibrous and anisotropic matrix (Ribero, Rubiolo, and Zorrilla 2007). Mozzarella cheese from bovine milk is a commodity produced, transformed and consumed worldwide. High value products are also produced using both water buffalo or cow milk, and in some of these cases, the production is controlled by Protected Designation of Origin (PDO) regulations in EU (Mozzarella di Bufala Campana; Mozzarella di Gioia del Colle) (European Commission 2020). Mozzarella cheese is obtained both by means of starter acidification or direct acidification with organic acids (Mucchetti, Pugliese, and Paciulli 2017). Bovine Mozzarella cheese is produced and categorized in two different groups (United States Food and Drug Administration 2019): High Moisture (HM) Mozzarella cheese (moisture content higher than 52% w/w but lower than 60% w/w) and Low Moisture (LM) Mozzarella cheese (moisture content between 45% and 52% w/w). The latter shows longer shelf life, and in various forms it is largely used as an ingredient of pizza and other preparations because of its unique functional properties such as meltability and stretchability (Bertola et al. 1996a). This product may also be modified in composition, and in this case, be called pizza cheese or cheese analog (Codex Alimentarius Commission 1978). US HM Mozzarella cheese has a moisture content lower than Italian Mozzarella cheese, usually ranging from 58 to 65%, with the latter that is stored and commercialized into a covering liquid to prevent moisture losses from the product (Mucchetti, Pugliese, and Paciulli 2017).

LM Mozzarella cheese is usually produced into blocks, ripened for a limited period (e.g. 2 weeks), and often further processed, into slices, cubed or shredded. LM Mozzarella cheese can be directly obtained by fluid milk renneting and/or by semi-finished curds that are stored and transported to the dairy to be stretched (Dalla Riva et al. 2017). On the contrary, HM Mozzarella cheese is a soft cheese, which is consumed fresh, and it can be produced in different shapes and sizes, sold in a covering liquid generally made of water, NaCl and organic acids (Mucchetti, Pugliese, and Paciulli 2017). The covering liquid has the function to avoid moisture losses and the formation of the rind in the outer part of the cheese. Because of the high moisture content and its texture, HM Mozzarella cheese is not usually suitable to be industrially shredded to be used in industrial pizza or baking preparations

despite a patent to perform this process when the cheese is in the frozen state has been reported (Coker et al. 2017).

Freezing of Mozzarella and pasta filata cheeses manufactured for pizza and baked foods production may be beneficial to extend shelf life (Pilcher and Kindstedt 1990). Immediately after stretching, salting and cooling, the cheese structure requires some aging period (a few weeks) before developing the desired functional properties, such as meltability, stretchability, melting color, fat release. However, after reaching the desired characteristics, Mozzarella cheese then rapidly loses these properties during additional storage time (Kindstedt and Fox 1993). Several literature studies evaluated the possibility to apply the freezing process to LM Mozzarella cheese by assessing the change in the rheological and melting properties of the cheese, as these are key quality attributes for the product. It has been reported that textural properties do not change significantly after short-term freezing (Cervantes, Lund, and Olson 1983); however, the product shows higher values of hardness and adhesiveness after 3 months of frozen storage at -20°C (Bertola et al. 1996b).

LM Mozzarella, refrigerated and frozen (blast frozen at -30°C for 4.5 h, stored at -20° up to 90 days) dynamic rheological properties showed that while LM Mozzarella stored under refrigerated conditions showed a softening of its structure because of the activity of endogenous proteases on α_{s1} and β-caseins, frozen and thawed cheeses had an increase in elastic modulus (Diefes, Rizvi, and Bartsch 1993). This was also reported by others (Bertola et al. 1996b; Reid and Yan 2004) also for other cheeses (Alvarenga, Canada, and Sousa 2011; Alberini, Miccolo, and Rubiolo 2015). The increase in elasticity and hardness compared to fresh control cheese has been attributed to dehydration of the protein network. After thawing, the dense protein network is no longer able to re-adsorb bulk water, diminishing its lubricant effect and thus producing a harder cheese structure (Bertola et al. 1996b). A different study (W. H. V. Chaves and Grosso 1999) showed no effect of freezing after 15-days frozen storage and 29-days tempering time after thawing, for cheese with low moisture and a high salt content (44.3% and 1.45%, respectively). The composition of this cheese may have limited the residual enzymes' activity. Other authors also attributed to the increase in elasticity the increase in stretchability and decrease in meltability measured in frozen mozzarella cheese (Oberg et al. 1992). Deep freezing of mozzarella in brine solutions has also been tested (Ribero, Rubiolo, and Zorrilla 2007), and dehydration of the protein matrix seems to be reduced in LM Mozzarella cheese, with better preservation of the rheological properties. Microstructure analysis revealed large voids between protein fibers in frozen Mozzarella due to protein dehydration and ice

crystals formation and growth (Graiver, Zaritzky, and Califano 2004; Kuo and Gunasekaran 2009), and after defrosting the structure appears more porous due to the presence of large serum pockets.

The effect of freezing in mozzarella also depends on residual enzymatic activity. During freezing, damage can be caused to the casein network and to the LAB starter cells by the ice crystals; residual proteolytic enzymes will lead to a higher degree of proteolysis promoting a lowering of textural and rheological properties (Graiver, Zaritzky, and Califano 2004). Moreover, freezing damage to the matrix can make it more susceptible to subsequent proteolysis after thawing; non-protein nitrogen and soluble nitrogen at pH 4.6 are higher for frozen-thawed samples already at the initial time of ripening (Graiver, Zaritzky, and Califano 2004). It has been reported that the non-protein nitrogen content of frozen stored LM Mozzarella cheese ripened for 6 days before freezing is higher than the refrigerated control (Bertola et al. 1996b), highlighting a significant interaction between freezing, storage time and proteolysis (Alinovi et al., forthcoming). Also, temperature abuses and freeze–thaw cycles will further affect proteolysis. The organic acid content, important in defining some of the flavor characteristics of the product, is similar in frozen LM Mozzarella cheese (at -20°C and stored for 6 days) if compared with the refrigerated control during a subsequently overall ripening period of 41 days (Califano and Bevilacqua 1999).

Functional properties are a direct consequence of the rheological characteristics of the product. Conflicting results have been published, possibly because of differences in composition, processing history, and freezing process. Kuo and Gunasekaran (2003) stated that modifications in cheese structure during freezing and frozen storage increased the meltability and decreased the stretchability of LM Mozzarella cheese, as a consequence of protein damage. Bertola et al. (1996b) studied the effect of freezing conditions, frozen storage, ripening and aging time on functional properties of LM Mozzarella cheese such as free oil, meltability and apparent viscosity and they found out that, under their particular conditions (-20°C in slow or fast conditions, using a cold chamber or ethylene glycol, respectively), they did not observe significant quality losses. Bunker (2016) did not observe modifications of these parameters as a function of the different freezing process tested (still-air freezing, air-blast freezing, immersion freezing at -30°C and immersion freezing with ethanol at -70°C). Reid and Yan (2004) observed that mozzarella frozen and stored at -28°C for 3 months, an increase in stretchability and decrease in meltability, also confirming results reported by Oberg et al. (1992). Apostolopoulos and Marshall (1991) showed that the freezing of LM Mozzarella cheese did not have a significant effect on meltability but on the amount of free oil that was significantly higher.

On the contrary, others (Diefes, Rizvi, and Bartsch 1993) reported a decrease of 14.7% of free oil for frozen stored LM Mozzarella cheese. Surface browning of frozen Mozzarella cheese was reported to be the equal (Oberg et al., 1992) or higher (Bunker 2016) than the control treatment.

The lack of homogeneity in results reported by these studies concerning LM Mozzarella cheese properties can be due to the different freezing processes (e.g. freezing temperatures; cheese size) and equipment used but also to the different cheese manufacture processes, composition and ingredient history (Alvarenga et al. 2013); in particular, Graiver, Zaritzky, and Califano (2004) applied a higher freezing temperature than Diefes, Rizvi, and Bartsch (1993) (-20°C vs. -30°C) that could promote the formation of bigger ice crystals causing higher damage to the cheese structure. Most studies lacked an in-depth proteomic characterization, and it may be hypothesized that partial hydrolysis of the proteins may affect the structure of the protein network and functionalities such as stretching, melting and elasticity, that are critical when the cheese is used as food ingredient (e.g. pizza).

It has been suggested that a period of tempering before or after freezing, may be necessary to soften the structure and recover optimal functional properties; while frozen storage leads to a more dehydrated protein matrix, with bigger serum channels and cracks as a consequence of ice crystallization, refrigerated storage acts in the opposite way, leading to a denser matrix because of water absorption and proteins swelling (Laurienzo et al. 2008; Ribero, Rubiolo, and Zorrilla 2009).

LM Mozzarella cheese freezing process can be applied either to whole blocks of cheese of different dimensions and shapes or to shredded cheese. In many food applications, Mozzarella is used in a shredded form and therefore many dairies shred the product prior to distribution to improve the ease of use. The higher freezing rate due to the small dimensions of the shredded cheese has an effect on the characteristics of the product: meltability and stretchability of the cheese will differ depending if the product was shredded or not, probably because of the higher freezing rate and the consequently lower damage to the cheese protein network (Oberg et al. 1992). Shredded cheese should be processed by applying a fluidized bed IQF method to prevent particles agglomeration; the frozen product can be used for pizza baking in frozen state or after thawing (Kielsmeier, Barz, and Allen 1990); a low air temperature (-35/-45°C) causes a fast freezing and a high, fast temperature decrease in the outer part of the cheese piece, promoting the formation of a crust, icy layer that prevents agglomeration. In this case, however, anticaking agents such as starch or whey powder may

be added, to prevent agglomeration during the process, during storage, and prevents caking during defrosting (Mccadam 1965; Coker et al. 2017).

Low resolution Nuclear Magnetic Resonance (NMR) spectroscopy and Magnetic Resonance Imaging (MRI) were applied to investigate water distribution and molecular mobility in frozen mozzarella (Kuo, Anderson, and Gunasekaran 2003). The water diffusion coefficient (D) increases with freezing at -21.5°C and after frozen storage for 1 or 4 weeks, and this change is attributed to the already described modifications of the protein matrix. The water is then less entrapped in the matrix. T2 relaxation times were shifted to lower values and the population distribution was narrower, pointing to the exchange of water molecules between phases with different mobilities. During tempering after thawing, a decrease in D values, an increase of T2 relaxation times and a broadening of the peaks was observed, suggesting that the cheeses may be able to partly regain their initial state (Kuo, Anderson, and Gunasekaran 2003). These studies indicated that NMR may be a powerful technique to determine changes and quality of mozzarella cheese after freezing; however, more work should be carried out to fully interpret these dynamics within the complexity of the processing, storage history and anisotropy of the structures.

Freezing of Italian HM Mozzarella cheese has also been studied, although information in this case is more limited. Conventional freezing was compared to blast freezing for Mozzarella of two different sizes (Conte et al. 2017) for 2 months of storage, using microtomography and sensory analysis. The results indicated that frozen storage decreases the pores dimension in the gel. The decrease in pore volume can be due to protein swelling and gel network rearrangements that can then fill the voids and change the microstructure of the sample. This is in agreement with the weight gain due to water absorption observed during refrigerated storage in HM Mozzarella stored in liquid (Laurienzo et al. 2008; Ribero, Rubiolo, and Zorrilla 2009). A gradual decrease in overall sensory quality is noted, quickly reaching a critical value of acceptability (59-60 days for 30 g size, 25-27 days for 250 g size).

Despite many authors studied the effects of frozen storage, no information is available on the effects of long frozen storage periods on the characteristics of Mozzarella cheese (e.g. more than 6 months). Moreover, for fresh products such as HM Mozzarella cheese, no studies are available on the physical and chemical properties after thawing. It may be possible to hypothesize that the shelf life is lowered by the modifications caused by freezing and prolonged storage.

3.8 Freezing of non-bovine cheeses: caprine and ovine milk cheeses

Freezing can be used as a solution to seasonality for high value cheeses, especially those produced from small ruminants' milk (Alvarenga et al. 2013). The product is frozen immediately after production (Casla et al. 1995; Fontecha et al. 1994) or after being fully ripened (Tejada et al. 2000; Tejada et al. 2002), depending on the type of cheese.

The effect of frozen storage (2 days, 3, 6 months at -20°C) of soft cheeses from ewe's milk before ripening period (28 days) has been reported (Van Hekken, Tunick, and Park 2005). Proteolysis slightly varied with control cheese showing higher concentration of peptides in the molecular weight range between (22.5-15 kDa), mostly assigned to β-casein hydrolysis; only the short-term frozen storage slightly modified the structure of the cheese, by reducing the elastic behavior of the body. It is important to note that the final pH values of the cheeses were in the range between 4.05 and 4.14, and such low pH value causes a significant reduction in neutral enzymes activity (e.g. plasmin). Martín-Hernández et al. (1990) evaluated the effect of freezing (-40°C using a plate freezer) and frozen storage (4 months) on four different caprine cheeses: a rennet-coagulated fresh cheese, a washed curd cheese made with a partial substitution of a part of the whey with 5% NaCl solution, a soft cheese made with surface flora (*Penicillium candidum* spores) and a semi-hard, Majorero-type cheese; all these cheeses were made using pasteurized milk with the addition of animal rennet and, with the exception of the rennet-coagulated fresh cheese, with addition of LAB cultures. In all the cases, with the exception of the rennet-coagulated fresh cheese, free nitrogen values were significantly higher in the frozen cheeses than their respectively controls, after the refrigerated ripening time. The higher proteolysis was related to the presence of enzymes from microbial lysis. A lower level of α_s-casein breakdown was measured in the frozen-stored cheeses and was attributed to the lower residual rennet (animal extract) activity, and to a decrease in protein accessibility due to the rearrangements in the network (Martín-Hernández et al. 1990). Lipolysis was not influenced by the frozen storage in all the cases, with the exception of the soft cheese with surface flora; in this case, frozen cheese showed a higher free fatty acids index. Sensory analysis demonstrated that the structure was acceptable in all the cheeses with the exception of frozen fresh cheese, due to a higher brittleness and graininess compared to control. The rennet-coagulated fresh cheese, with a moisture content of about 60% showed very little freezing stability.

Freezing of soft goat cheeses (-20°C) and subsequent refrigerated storage (14 and 28 days) affect the organic acids composition as a result of different bacterial fermentation during ripening (Park and

Drake 2005; Park and Lee 2006). However, no differences in pH and lipolysis were noted. Despite the change in organic acids, sensory perception of goat cheese has not been shown to be different after the frozen storage period in these high moisture and mild cheeses. These results were confirmed by a following work that showed that freezing (-20°C for 24 h) followed by ripening for 30 days did not impact over the majority of flavor attributes with the exception of waxy/goaty and brothy flavors that decreased and increased, respectively following 3-6 months of frozen storage (Park, Gerard, and Drake 2006); overall freshness was largely unaffected. Longer storage, up to 5 years, caused significant reduction of freshness perception of caprine cheese (Park 2013).

Storage studies on semi hard ewe's milk cheeses have also been reported (Fontecha, Bellanato, and Juarez 1993; Fontecha et al. 1994; Fontecha et al. 1996). The effect of freezing (-35°C and -80°C using a plate freezer and liquid nitrogen, respectively) and frozen storage (-20°C for 4 months) of young semi hard ewe's cheese promoted an increase in proteolysis during the following ripening (up to 90 days at 11°C), as already evidenced in the case of caprine cheeses (Martín-Hernández et al. 1990); WHC and a_w values in frozen/thawed cheeses were respectively higher and lower because of the more pronounced proteolytic phenomena. Proteolysis caused the release of low molecular weight peptides that lowered the aw and the formation of ionic groups, that increased the WHC by bonding the free water. This observation is related to the presence of residual viable microbial population after storage. The caseinolytic and aminopeptidase activity of *L. lactis subsp, lactis* and *L. lactis subsp, cremoris* is affected by frozen storage but not by the freezing process itself (Casla et al. 1995). Changes in proteolysis were not influenced by the different freezing methods. However, slower freezing rates combined with prolonged frozen storage (4 months) were responsible for the presence of cracks and voids in the frozen cheeses as also reported in the case of Mozzarella cheese (Graiver, Zaritzky, and Califano 2004; Kuo and Gunasekaran 2009), that caused also a softening of cheese body already after only 2 days of ripening (Fontecha et al. 1996; Fontecha et al. 1994).

Near Infrared and Raman spectra of frozen, non-ripened cheese samples highlight an increase of protein unordered structure following freezing, while control cheeses show a higher proportion of β-sheet and α-helix structures (Fontecha, Bellanato, and Juarez 1993); frozen storage does not seem to cause additional changes. The same work also demonstrated that two different freezing methods cause differences in the spectra of cheeses during ripening, with the young cheese frozen at -35°C showing a larger development of unordered structure than the young cheese frozen at -80°C using

liquid nitrogen. This difference could be due to the larger ice crystals formation and the slower freezing rate as well as an increased proteolysis (Fontecha, Bellanato, and Juarez 1993).

Tejada et al. (2000, 2002) evaluated the effect of freezing (-20°C and -82°C) and prolonged frozen storage (up to 9 months) on the characteristics of a ewe's milk cheese manufactured using a vegetable coagulant; differently than the previously mentioned studies, these works applied the freezing process of the cheeses ready for consumption after a 90-days ripening period and were analyzed after thawing. Chemical analyses did not show any significant and large difference, in agreement with Prados et al. (2006) that studied the effect of freezing and 9-months frozen storage on characteristics of fully ripened Manchego-type cheese. Moreover, the number, size and distribution of the eyes were significantly reduced after 90 days of storage in freezing conditions, probably due to the destruction of the structure, similarly to what reported by Conte et al. (2017).

Alvarenga, Canada, and Sousa (2011) evaluated the effect of freezing (-20°C and -30°C) after 28, 35 and 42 days of ripening and frozen storage (12 months at -10 and -20°C) on Serpa cheese. While the primary proteolysis was blocked by frozen storage, the secondary proteolysis still proceeded, as frozen cheeses had higher values of non-protein nitrogen. Physical analyses of the cheese showed an increase in hardness after frozen storage, as previously reported by Diefes, Rizvi, and Bartsch (1993).

3.9 Freezing of other cheeses

Moisture, fat and salt content play a critical role in defining the physical chemical and sensory characteristics of frozen Cheddar cheese (Kasprzak, Wendorff, and Chen 1994). Firmness decreased after freezing/thawing when the moisture content was between 35-44% as a consequence of ice crystals damage. This was also confirmed with textural and microstructural observations in another study (Reid and Yan 2004). Proteolytic activities continue during storage at -18°C, and the rate of proteolysis further increases after thawing due to rearrangements in the protein network and release of enzymes from ruptured bacterial cells. Meltability and texture are not significantly affected after freezing (Reid and Yan 2004). It has been reported that cheeses with a moisture in fat-free cheese between 55-57%, corresponding to the firm semi-hard designation of Codex Alimentarius general cheese standard 283-1978 (Codex Alimentarius Commission 1978), are the most suitable to be frozen, as determined a higher degree of textural acceptability (Kasprzak, Wendorff, and Chen 1994).

Simov and Ivanov (2005)studied the effect of freezing (at -16°C in still air freezing conditions) and frozen storage (-10°C/-12°C up to 12 months) of young, semi ripened and ripened Kashkaval cheeses. In this case also proteolysis of the cheese was significantly delayed but not arrested by frozen storage. The highest increase in non-casein and non-protein nitrogen was observed in young frozen cheeses, because of the higher pH value, which favors the activity of enzymes during the step of ripening after thawing in comparison with the control. A similar trend was also reported for a soft, semi cooked, short ripened cheese like Port Salut Argentino cheese (Verdini and Rubiolo 2002), that showed a higher ripening index and higher concentration of hydrophobic peptides also when immediately analyzed after thawing because high residual rennet activity and the presence of peptidases from damaged LAB cells (Verdini, Zorrilla, and Rubiolo 2005).

On the contrary freezing may be applied to stop mold induced proteolysis of ripened blue cheese, reducing the rate of aging during shelf life or to store young cheeses in order to mitigate the effects of seasonal variations in milk availability. Freezing of unripened Cabrales cheese (-40°C using a plate freezer) delayed mold growth and the rate of primary and secondary proteolysis during the subsequent 8-months ripening of thawed cheeses as a consequence of freezing damages (Ramos et al. 1987).

Freezing may become a solution to improving shelf life of fresh, unripened cheese. However, high moisture content presents challenges as freezing may result in a detrimental effect of cheese body and texture, as observed for Italian HM Mozzarella cheese. This was shown for example, for freezing

of Cottage cheese (D. R. Emmons, Beckett, and Tape 1968), which results in a mealy, fragile appearance after thawing, with extensive whey separation. Reformulation may be needed to improve the freezing stability of such products. Also, in the case of cream cheeses, freezing is industrially applied as method to improve storability and convenience. To the date, there is scarcity of literature data about the effects of freezing and thawing on fresh cheeses characteristics, despite these are industrial common methods used to improve shelf life of the cheeses. In cream cheese products, to preserve proteins functionality and structure, as well as cheese characteristics, and to limit denaturation, different cryoprotectants and stabilizers have been evaluated and used; also, antioxidants can be incorporated to minimize oxidative reactions against proteins and fat during storage. A reduction of freezing-induced modifications can be achieved by incorporation of sorbitol (typically 4% each), polyphosphate, glycoproteins, polysaccharides, polyols, sucrose and other sugars (Dey et al. 2019; Xiong 1997).

3.10 Freezing of cheese curd

Curd is the intermediate product of rennet or acid coagulation milk, that is obtained after whey drainage. Curd is industrially produced as a semi-finished product and it is immediately packaged with the aim of regularly supplying cheesemaking sites lowering the costs and volumes that are involved in milk transportation (Barone et al. 2017a; Barone et al. 2017b; Barone et al. 2017c). Freezing of curd may be preferred to refrigerated storage of curd to extend the shelf life of this kind of semi-finished product (Pazzola et al. 2013). Moreover, curd's freezing can be an alternative to milk's freezing, as it reduces the warehousing needs and allows first step of manufacturing to occur closer to the milk production sites. Freezing of curds will therefore be an opportunity to delocalize cheesemaking in production areas closer to the end user, trading an intermediate product, and allowing to produce on demand, fresh product closer to the consumer, for example, in-store. Furthermore, cheese curd freezing may a be a tool for controlling milk's surplus by increasing storability.

Curd's freezing is applied to promote cheese productions that are seasonally limited in term of quantity (caprine, sheep, buffalo milk) (Lu and Miller 2019) or in the case of cow's milk, frozen curds are mainly used as an ingredient for producing pasta filata and/or processed cheeses.

An important parameter that has to be considered in relation with the cheese to be produced, is the pH of frozen cheese curds. The pH at the end of the coagulation process should be in the range between 6.3–6.6 if the type of cheese requires a rennet-type coagulation, while in the case of cheeses characterized by acid-type coagulation, the pH of the curd should reach the isoelectric point of caseins after lactic acidification processes (range 4.6–4.9) (Belitz, Grosch, and Schieberle 2009; Hori 1982). In industrial processes milk acidification made by LAB can be integrated or substituted by the addition of acidulants (e.g. lactic, citric acid, glucono delta lactone); this step can affect the amount of residual enzymes in the curd, and helps to control pH during thawing (Bansal, Fox, and Mcsweeney 2007). The type of acidification applied to modify the pH value of the frozen curd is of particular importance for products intended for pasta-filata cheese production, as it regulates the degree of demineralization of casein (calcium removal), that is one of the main factors determining stretching behavior and final textural properties of the cheese (Sheehan and Guinee 2004). Generally, curd's pH is kept slightly higher than the desired pH for the next technological operation (i.e. curd's stretching in the case of Mozzarella cheese) to avoid over-acidification (Vélez et al. 2015).

Also, the draining process may be an important step in the production of curds to be frozen, as it regulates the amount of water to be frozen and thus the time required for process, and the properties of the curd as an ingredient for further processing. Whey drainage can be obtained by the pressing or, in the case of fresh acid coagulated cheeses, traditionally by natural draining that can last up to 12-15 h until a dry matter content around 40% is reached (Portmann 1970) or actually by separation or membrane filtration (Mucchetti et al. 2000). pH and moisture content are critical parameters that have to accurately decided before performing freezing, as high values of these two parameters create favorable conditions for proteolytic or lipolytic activities (Alonso et al. 2011; Picon, Gaya, et al. 2010). The size of the curds may also be critical depending on the process.

The degree of integrity of casein is an important factor that has to be defined in view of the functional properties of the final cheese to be produced; highly hydrolyzed casein chains cause generally low water absorption, while more preserved and intact casein proteins maximize curd's attitude to be stretched and increase water absorption in the case of pasta filata type cheeses (Barbieri et al. 2014). Barone et al. (2017b) showed that deep-frozen cow's curds stored at -18°C had a lower protein degradation even compared to refrigerated curds after a short shelf life period; this feature makes frozen curd a better ingredient than refrigerated curd for high moisture products. However, long frozen shelf life causes a softening of the casein structure, because of the residual activity of proteases and physical damage of ice crystals, according to phenomena occurring in frozen cheeses.

To minimize curd's softening during storage and improve storability, renneting agents characterized by a low non-specific proteolytic activity, as camel chymosin (Alinovi et al. 2018) and thermophilic starters with a low proteolytic activity should be preferred.

However, as well as in the case of Mozzarella cheese intended for pizza production, a tempering period may be required before further processing, if the body is too firm, to reach optimal functional properties and best final product quality. In fact, as in the case of cheeses during frozen storage, the modification of protein structure and the redistribution of the water molecules within the protein network can lead to a hard curd structure (Kljajevic et al. 2017).

Several research studies evaluated the applicability of frozen curds for the manufacture of different kind of cheeses.

Hori (1982) studied the feasibility of the freezing and thawing processes to store curds intended for cream cheese production; curds were treated by applying 5 different freezing (still air at -20°C and -

80°C, CO_2 at -80°C, N_2 vapors at -196°C and ethanol immersion at -80°C) and thawing processes (still air at 0°C and 12°C, water immersion at 7°C, hot water immersion at 50°C and 80°C), corresponding to different freezing and thawing rates. Results showed an increase in textural hardness with decreasing freezing rate and with increasing thawing rate, respectively (Hori 1982). Textural results were in accordance with NMR T_2 relaxation time of bound water, that followed the same trend described for textural parameters, giving information about the extent of freezing and thawing injury, that were maximum at the lowest freezing and thawing rates, with a larger effect of thawing than freezing (Hori 1982).

While the increasing freezing damage is an expected result with the lower freezing rate, it is not always true with lower thawing rate. In fact, while slow thawing enhances the rate of physical and chemical modifications that occur in the phase transition temperature range, it has been reported that slow thawing may have beneficial effects to the structure, for example, re-equilibration of minerals (in particular calcium and phosphorous) and the reabsorption of unbound water (Haard 1997). In fact, the amount of bound water decreased with the decreasing freezing rate and increased with the decreasing thawing rate.

A process for preparing Mozzarella cheese from single deep frozen curd by IQF technique without the need of any fresh curd has been patented (Zambrini and Bernardi 2017); the curd of appropriate size (longest side smaller than 10 cm, preferably 2 cm) can be individually frozen (IQF) and stored for 1 year or more. Curd portions can be thawed in line using water-steam used for steam stretching or with the aid of microwave or dielectric heating; the final cheese product can be obtained with only marginal mass losses and without the production of any by-product if the correct amount of added water is fully adsorbed by the curd during stretching (Zambrini and Bernardi 2017).

Frozen and thawed curds can also be used to produce fresh cheese to be further ripen; it has been reported that freezing applied to curd after draining can lead to a better-quality product than for ripened cheese (Portmann 1970). The presence and activity of bacteria and enzymes is of key importance to develop the optimal cheese texture and flavor during ripening. For example, Vélez et al. (2015) demonstrated, with a mini-curd model of young hard-cooked cheese (i.e. Reggianito Argentino) that no significant differences were found in pH, temperature during manufacture and composition between the cheese manufactured using frozen curd and the fresh control. As the curd for this kind of cheese is typically rennet-type, the procedure involved the standardization of pH at 6.40 by means of lactic acid and the addition of the commercial starter; the curd particles are stored

frozen stored (-18°C) before the acidification process is carried out later, after thawing, until a pH of 5.6 is reached.

In the case of caprine curds, Sendra et al. (1999), did not observe significant changes in the main components of sheep's milk cheese made with frozen curd or in the lipolytic activity. Moreover, temperature variations associated to curd's frozen storage did not cause a significant change in composition, proteolysis, lipolysis or fat oxidation, and pH; only changes in microstructure were observed in frozen curds, but were partially overcame by the ripening process of the cheese (Sendra et al. 2002). On the contrary, other authors (Alichanidis et al. 1981) demonstrated poor quality for cheese (Teleme cheese) made by frozen curd (-40°C per 12 h), stored at -20°C up to 6 months, due to extensive proteolysis, accelerated ripening, a lower water holding and moisture content and a crumbly texture. A higher pH of the frozen-curd and a higher extent of proteolysis was the cause for the quality challenges of the cheese made with frozen curd compared to control.

Several works studied the effect of freezing (-52°C) and frozen storage (-24°C for 4 months) on cheeses made by mixtures of frozen and fresh curds; in these works, goat's curd freezing and frozen storage was combined with the application of high-pressure treatments (Alonso et al. 2013; Alonso et al. 2011; Campos et al. 2011; Picon et al. 2013), pasteurization (Alonso et al. 2013), different curd's scalding times (Picon, Alonso, et al. 2010) and different pressing times (Picon, Gaya, et al. 2010) to observe possible differences during a 60-days ripening period of Hispánico cheese.

As frozen stored curds for cheesemaking are used by cheese producers to reduce production costs and increase margins, their application can be perceived as a negative factor by consumers in the case of traditional, high quality products. In particular, freezing and frozen storage of curds is not allowed for some PDO cheeses that have to follow EU regulations. Authors studied the possibility to trace the use of frozen stored curds in cheesemaking by measuring the presence of proteolytic markers generated by the residual activity of renneting agents and plasmin (Di Luccia et al. 2009; Manzo et al. 2017). Research studies have suggested that the presence of the fragment f(24-199) of α_{s1}-casein can be used as a marker to reveal the use of frozen stored curd in HM Mozzarella and in PDO Buffalo Mozzarella cheese making (Faccia et al. 2014; Petrella et al. 2015); plasmin activity against β-casein leads to the formation of γ_1, γ_2, γ_3-caseins fragments (f(29–209), f(106–209), and f(108–209)) in the case of bovine milk, while an additional fragment f(69–209) can be released and detected in the case of buffalo milk. Fragment f(24-199) of α_{s1}-casein is the primary proteolytic product generated by the residual activity of renneting agents and in particular calf chymosin. As

Mozzarella cheese curd temperature during stretching can inactivate most of chymosin present in the curd, a long term frozen storage of the frozen cheese curd before stretching can favor the formation of the fragment f(24-199) of α_{s1}-casein, that can be used as a molecular marker of the use of a frozen cheese curd (Faccia et al. 2014).

3.11 Conclusions and outlook

Freezing of dairy products is an effective method to extend shelf life and market reach both for high value products or for industrial products, such as cheese curds or mozzarella cheese. This is particularly important when biochemical and physical modifications due to ripening and/or storage need to be halted and when delocalizing cheese production, reaching long distance markets, controlling milk's seasonal availability and improving the organization of the dairies would be beneficial.

However, several factors need to be controlled to obtain products with desired characteristics and without textural and sensory defects. First and foremost, the physical properties of the product are modified by the formation of ice crystals, and water mobility is affected. This can create changes in the porosity of the protein networks, and rearrangements and aggregation of the milk proteins. The kinetics of freezing significantly impact the ice crystals size, and the amount of unfrozen water.

Due to salting out, there will be protein rearrangements, dehydration and the formation of aggregates of caseins. These chemical modifications can have significant detrimental effects on the textural, functional properties of the cheese or cheese curd (WHC, stretchability, hardness etc.). Despite the fact that the chemistry and texture changes caused by freezing were investigated for decades, a more in depth understanding of the proteomics is needed for a better understanding of the mechanisms behind the changes in the structure properties and improve their monitoring.

Freezing conditions industrially applied to dairy products can significantly increase the microbial mortality of selected species and strains; in the case of curds or young cheeses to be further ripened, this phenomenon can have important consequences in the development of flavors and texture during cheese aging. Moreover, ice crystal damage to the cell wall and membranes can improve the release of intracellular enzymes. The residual activity of those enzymes, together with that of the indigenous lipolytic and proteolytic enzymes present in milk, may also affect significantly the chemistry, flavor and texture properties of the cheese matrix. For this same reason, careful control of the quality of

the milk is needed. While during frozen state most reactions are delayed, enzymatic reactions can continue also at temperatures between 0 and -10°C, because of freezing-concentration phenomena that increase substrate accessibility counterbalancing the reduction of reaction rate due to the lower temperature. Storage at temperatures close to this critical range can significantly affect the quality of the frozen product. Modification of protein supramolecular structures with freezing, can improve the rate of biochemical reactions in particular after thawing and subsequent refrigerated storage, as a consequence of a more accessible structure (e.g. cleavage sites). Tempering or ripening periods have to be re-evaluated when using frozen products. In case of curds or young cheeses to be further ripened, it should be noted that an accelerated aging should be expected with also some modifications of aroma and characteristics.

To avoid quality defects, freezing should be performed in a fast, well-controlled process to minimize crystal growth and freezing damage; most importantly, the temperature during frozen storage should be maintained as constant as possible to avoid melting and recrystallization phenomena, and as low as possible, to slow down enzymatic reactions by lowering the aw. Cheese and curd composition and characteristics (e.g. pH, moisture content, percentage of components etc.) should be optimized in order to obtain the best results; product's size should be small as possible to lower the freezing time. Proper packaging material have to be used to avoid moisture losses and to prevent oxidative reactions.

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3.14 Figures

Figure 1. State diagram of an aqueous solution at constant pressure. T_f, T_s delineate the freezing point and solubility curves as a function of total solids. T_g is the glass transition curve, again as a function of solids concentration. T_e is the eutectic point, $T_{g'}$ is the maximal glass transition temperature of the supersaturated solution. Dashed lines indicate metastable equilibriums.

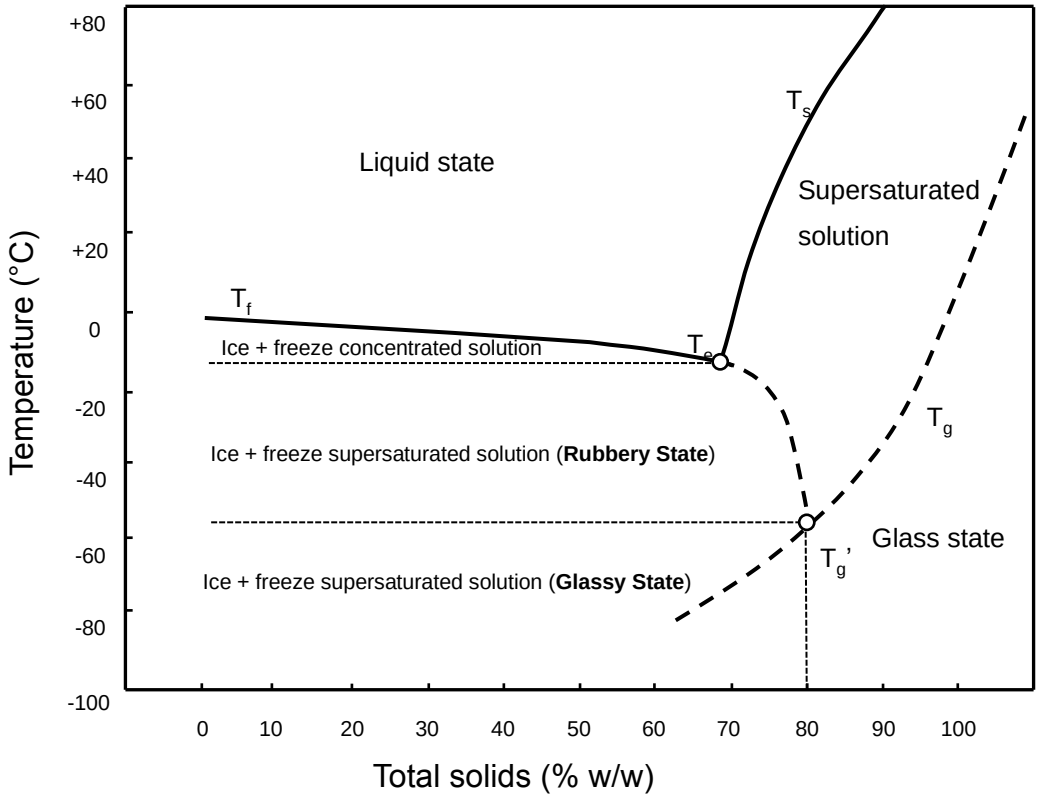


Figure 2. Two-dimensional, schematic representation of the freezing process of curd and cheese, indicating heat and mass flows across the product's freezing front. T_{ext} is the temperature of the external ambient, T_m is the melting point temperature of the product, T_f is the temperature of the frozen region of the product, T_u is the temperature of the unfrozen region.

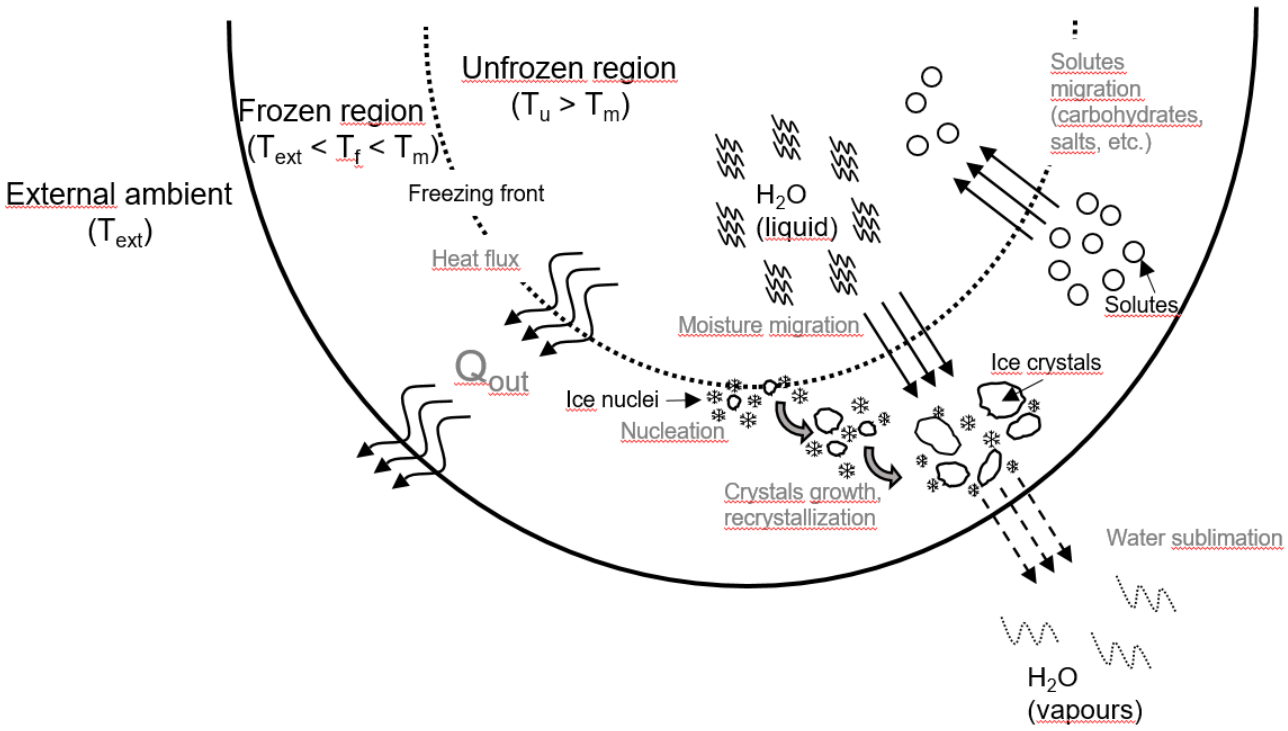
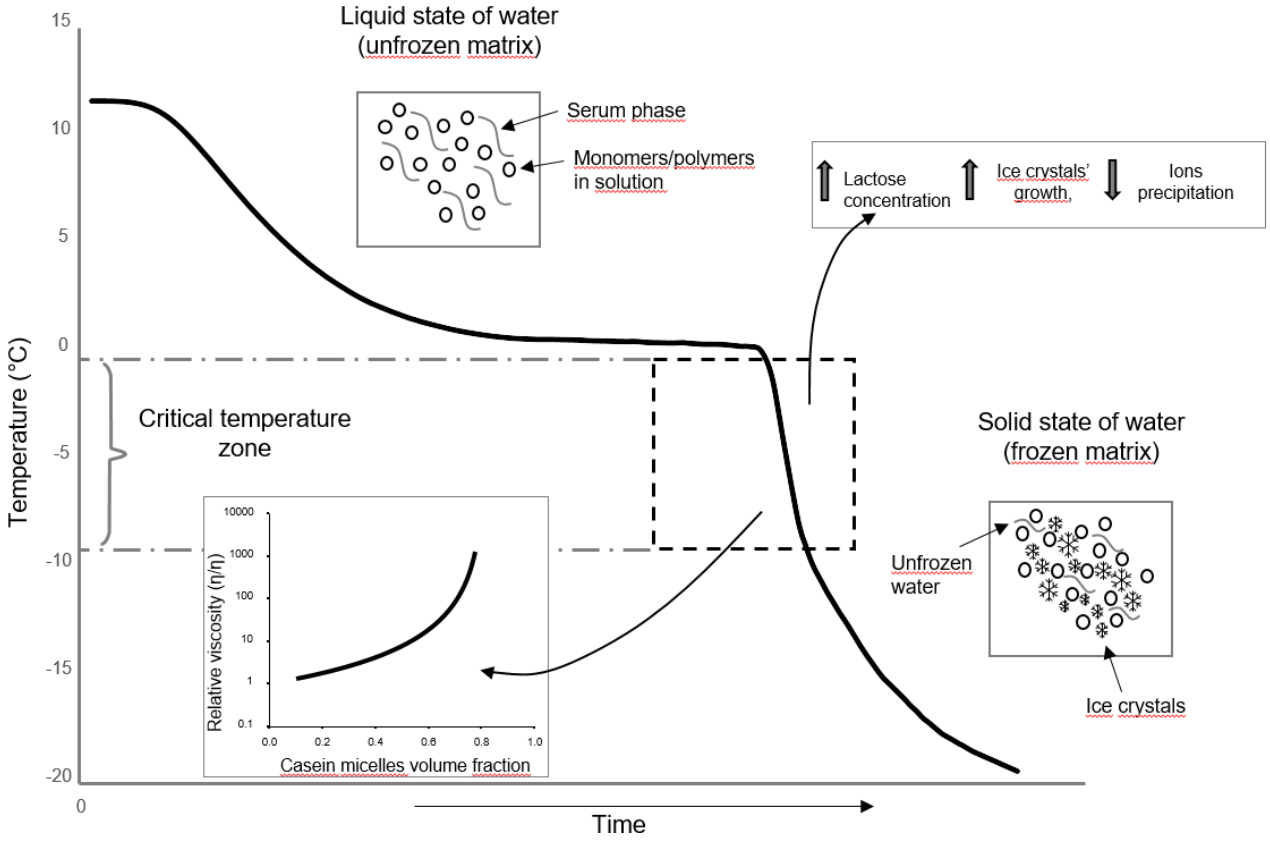


Figure 3. Schematic representation of temperature time freezing curve and physical modifications that occur during phase change.



4. A coupled photogrammetric - finite element method approach to model irregular shape product freezing: Mozzarella cheese case

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4.1 Abstract

The freezing process can be industrially performed to extend shelf life and to improve exportability of Italian high-moisture Mozzarella cheese. This cheese is usually characterized by a non-regular spheroidal shape that may be responsible for local differences of temperature on the surface and that can influence the overall freezing time. In this work, Mozzarella freezing was modelled by coupling the finite element method and a photogrammetric procedure that permitted to reconstruct the three-dimensional domain of the product. Computational models were validated by performing experimental trials, and results were accurate. With the photogrammetric technique it was possible to estimate volume, surface area, shape and size of the cheeses, and to study temperature-surface distribution that was found to be non-homogeneous. Freezing models highlighted that the surface area-to-volume ratio of the product is one of the most critical parameters that define the freezing time of cheese. A geometrical approximation of the cheese based on the surface area-to-volume ratio, showed good accuracy in terms of freezing times. These models can be valuable for Mozzarella cheese freezing optimization and design, to recover efficiency and to improve quality.

Keywords: computational modelling; freezing process; irregular geometry; photogrammetry; Mozzarella cheese; FEM analysis

4.2 Introduction

High-moisture (HM) Mozzarella cheese is an Italian fresh cheese characterized by a moisture content usually higher than 60%, stored into covering liquid and exported worldwide (Mucchetti, Pugliese, & Paciulli, 2017). Mozzarella overseas export increased by 16.4% from 2016 to 2017, and extra-European countries imported more than 30% of the total amount of exported cheese (CLAL, 2020).

The short shelf life of this cheese (usually lower than 30 days), forced cheese manufacturers to use airplane instead of ships for exportation. In this context, freezing can help to preserve Italian HM Mozzarella cheese freshness for several months (Conte et al., 2017), allowing to choose more economical transports (Estrada-Flores, 2016). Nowadays, Italian HM Mozzarella cheese freezing process is performed in industrial scale based on empirical knowledge. As freezing is a highly energy-consuming process, mathematical modelling could be useful to optimize, scale and control the industrial process.

Surface peeling is one of the problems limiting the shelf life of the cheese during refrigerated storage when immersed in a covering liquid (Laurienzo et al., 2008). However, peeling can also occur after thawing of the frozen product. Local differences of temperature on the cheese surface because of its non-regular shape can be supposed as a contributing factor to surface cracking during freezing (Reid & Yan, 2004). Mozzarella cheese is produced in a multitude of shapes by pushing the stretched curd into cavities located on a rotating cylinder and then chilling the molded cheese in fresh tap water so to preserve the shape. The most famous one is the spheroidal shape. However, because HM Mozzarella cheese is a viscoelastic solid, unavoidable mechanical stresses (weight force, during immersion chilling, belt transportation and packaging), can cause an irreversible deformation of the cheese shape to a non-regular ellipsoidal shape.

Determining the shape and the volume of a product is an important aspect in industrial quality control of various food products (Olatunji, Love, Shim, & East, 2019). In recent years, photogrammetric technique has been successfully applied in food and animal sciences to accurately predict volume and morphological properties of irregular shaped objects (Syngelaki et al., 2018; Urso & Marino, 2016; Waite, Schrader, Mellish, & Horning, 2007; Wu et al., 2004; Zhang, Wu, Qiu, & He, 2016). This technique can have important industrial applications in food processing, as it can be easily implemented in in-line applications (Zhang et al., 2016).

The freezing process has been modelled in the last years for different food solid products such as mushrooms (Santos & Lespinard, 2011), marine products (Dima, Santos, Baron, Califano, & Zaritzky, 2014), butter (Nahid, Bronlund, Cleland, & Philpott, 2008), par-baked bread (Hamdami, Monteau, & Le Bail, 2004) and cheese slabs (Zorrilla & Rubiolo, 2005). Several studies concerning the development of empirical, analytical or numerical methods to predict the freezing process of irregular shaped foodstuffs have also been carried out by simplifying the three-dimensional, spatial domain of the product using shape factors or geometrical formulae (Cleland, Cleland, & Earle, 1987; Delgado & Sun, 2001; Hossain, Cleland, & Cleland, 1992; Pham, 2014). However, as in many of those cases, object domain is considered as a standard geometric shape (e.g., block, ellipsoid, and cylinder), models are not fully applicable to the case of irregular-shaped products (Cepeda, Weller, Thippareddi, Negahban, & Subbiah, 2013). Recently, Santos, Vampa, Califano, & Zaritzky (2010) developed freezing models of bakery products by closely reconstructing the geometrical domain of the product using a computer-aided design software. Moreover, computational models of cooling and heating processes involving non-regular shaped products were developed in the last years using three or two dimensional scanning techniques and image processing softwares (Cepeda et al., 2013; Fabbri, Cevoli, Alessandrini, & Romani, 2011; Lespinard, Goñi, Salgado, & Mascheroni, 2009).

The purpose of this study was to model the freezing process by means of a coupled photogrammetric - finite element method approach, that could permit an accurate reconstruction of the real geometrical domain of the cheese without geometrical approximations, and to investigate the influence of the non-regular shape of HM Mozzarella cheese on the local heat transfer.

4.3 Materials and Methods

4.3.1 Sample preparation and freezing experiments

Fresh HM Mozzarella cheese samples were kindly provided by Alival S.p.A. (Nuova Castelli S.p.A. RE, Italy) as individual 100 g-packaged portions (nominal weight) containing 100g of covering liquid (0.4 % w/w NaCl). Cheeses were stored at 4 ± 1°C for 5 days until the application of the freezing process. Freezing processes were performed using an air blast freezer (mod. MF25.1, Irinox, TV, Italy) characterized by a total volume of the freezing chamber of 0.5 m^3. Before freezing, cheeses were separated from the covering liquid and were placed on a HDPE net support characterized by a sparse squared mesh (side size = 0.02 m), in order to minimize the product-surface contact and the consequent effect on heat transfer; samples were equilibrated at an initial temperature (T_0) of 10.7 ± 1.5°C. Fourteen samples were processed per freezing cycle, until a temperature of -20°C in the core of the product was reached. Cheeses number and position into the freezing chamber was kept constant in all the trials in order to avoid a detectable impact of the chamber load in terms of process duration (minutes) (**Figure 1**).

Three freezing conditions (***Fc***), governed by air temperature and velocity into the freezing chamber, were considered: **C1**) -34.0 ± 2.4°C, 4.1 ± 0.6 m/s; **C2**) -30.0 ± 2.4°C, 2.5 ± 0.4 m/s; **C3**) -24.4 ± 1.5°C, 1.3 ± 0.2 m/s. Air velocity into the freezing chamber was recorded in the proximity of sample location by using a datalogger (mod. 435-2, Testo SE & Co. KGaA, Lenzkirch, Germany) equipped with a hot wire anemometer (mod. 0635 1535), while air temperature was acquired using a thermocouple K-type (Ni/Al–Ni/Cr); to validate the freezing models, temperatures of one sample per freezing cycle were monitored in a central location and in the outer part of the cheese using thermocouples connected to a multimeter acquisition system (mod. MV100, Yokogawa Electric Corporation, Tokyo, Japan). The temperature-monitored sample was always positioned in the same point of the freezing chamber in order to avoid possible effects related to a non-homogeneous distribution of air temperatures and velocities.

In order to build and validate the models, temperature of two Mozzarella cheeses (a and b samples) was experimentally acquired during independent freezing cycles for each of the applied freezing conditions (C1, C2, C3).

4.3.2 Geometrical domain of the cheese

4.3.2.1 Cheese geometry reconstruction based on the photogrammetric approach

After being frozen, cheeses that were considered for temperature acquisition, were photographed using a 12-megapixels smartphone digital camera (Moto g6 Plus, Motorola, Illinois, USA) having a 3.64-mm focal length, a camera stand that was fixed at a distance of 0.05 m from the object, a self-constructed turntable, a lighting equipment composed by two 60W lamps (Philips Softone, Amsterdam, The Netherlands), a white paper light diffusion system to eliminate shadows and a black background. Cheese samples were positioned on a cylindrical support (radius 0.01 m, height 0.025 m) painted by black at the center of the turntable, in order to acquire and model also the bottom surface of the cheese (**Figure 2**). While rotating the turntable, at least 60 photos were acquired at 3 different camera angles (-30°, 0°, +30°) with a resolution of 3120 × 4160 pixels, a focal ratio of 2, an exposure time of 1/30 s and an ISO sensitivity of 100 for each cheese (**Figure 3a**).

Acquired photos were in batch post-processed by applying contrast enhancement using XnConvert software (XnSoft, Reims, France). To correctly estimate the dimension and the shape of the object and to avoid geometric distortion and optical defects, prior to photogrammetric reconstruction, the camera was calibrated similarly to Zhang et al., (2016) using Agisoft Lens package (Agisoft, St. Petersburg, Russia). The software package uses a pinhole camera model for lens calibration and the Brown's distortion model to correct both tangential and radial distortion (1):

$$\begin{cases} x_u = (x_d - x_c)(1 + K_1 r^2 + K_2 r^4 + K_3 r^6) + P_2(r^2 + 2(x_d - x_c)^2) + 2P_1(x_d - x_c)(y_d - y_c) \\ y_u = (y_d - y_c)(1 + K_1 r^2 + K_2 r^4 + K_3 r^6) + P_1(r^2 + 2(y_d - y_c)^2) + 2P_2(x_d - x_c)(y_d - y_c) \\ r = \sqrt{(x_d - x_c)^2 + (y_d - y_c)^2} \end{cases} \quad (1)$$

where x_u, y_u are the undistorted image point coordinates projected by the camera model, x_d, y_d are the distorted image point coordinates, (x_c, y_c) is the distortion center, K_1, K_2, K_3 are the radial distortion coefficients, P_1, P_2 are the tangential distortion coefficients.

Projection point coordinates of the image (u, v) can then be calculated from (2):

$$\begin{cases} u = x_c + x_u f_x + \alpha y_u \\ v = y_c + y_u f_y \end{cases} \quad (2)$$

where f_x, f_y are the focal lengths and α is the skew coefficient between the x and the y axis.

After camera calibration, background was subtracted from all the photos and the 3D sparse and then dense point clouds of the cheeses were reconstructed using a commercial photogrammetric software (PhotoScan v. 1.2, Agisoft, St. Petersburg, Russia).

The point cloud of an object is the spatial information of the object's external surface; the cloud is calculated by a stereo matching procedure of the captured images, that consists in aligning the photos around the object's space and looking for common points (Syngelaki et al., 2018). Both sparse and dense point clouds were processed by the photogrammetric software using high-accuracy settings to build an accurate spatial model of the cheese (**Figure 3b**); the aligning process took about 7 min and 20 min, respectively for the sparse and dense point cloud, running on a Fujitsu Celsius Workstation (Tokyo, Japan) with a 3.5-GHz, 6-cores, Intel Xeon CPU and 32 GB RAM. To obtain the real dimensions of the object from the geometrical model, characteristics dimensions of the cheese (i.e. distance between two marked points on the product's surface) were also measured using a calliper and were inserted into the software.

The dense point cloud was then imported into Solidworks 2017 (Dassault Systèmes, Vélizy Villacoublay, France) and then transformed into a surface mesh at a degree of surface simplification of 90% to avoid meshing problems with rough surfaces (Zhang et al., 2016); as the generated surface mesh is not directly recognised by the Finite Element Method (FEM) package used, a surface solid domain was built (**Figure 3c**) using the automatic surface creation tool of ScanTo3D add-in. In this final step, the level of surface details was kept at the minimum level, as from preliminary trials it would not affect subsequent results obtained from the computational model. After the whole photogrammetric procedure that is summarized in **Figure 3e**, the created surface solid can be used to build cheese freezing models using a Finite Element Method (FEM) package.

4.3.2.2 Simple geometries based on regular solids

Different regular geometries were built using Solidworks 2017: a sphere, an oblate and a prolate ellipsoid (**Table 1**). This procedure allowed to compare the numerical results obtained with the photogrammetric reconstruction of the non-regular cheese shape, with the results obtained considering simplified and approximated geometries.

The sphere and the oblate ellipsoid were built a) by simplifying the volume to the nominal cheese mass (100.0 g) or b) by imposing the calculated volume from the real cheese mass measured using a laboratory scale (mod. BCE 5200, Orma, Milan, Italy) with an accuracy of ± 0.1 g.

The prolate ellipsoids were built also considering and approximating the estimated surface area-to-volume ratio (*SA:V*) of the cheeses, as further described in section 3.3.

As Mozzarella cheeses were not characterized by the presence of openings, cheese volume was approximated by applying equation 3 reported by Choi & Okos, (1986) predicting density on the base of cheese composition, and then equation 4:

$$\rho(T) = \frac{1}{\sum_{i=1}^{n} {x_i}/{\rho_i(T)}} \quad (3)$$

$$V(T) = \frac{m}{\rho(T)} \quad (4)$$

where $\rho(T)$ expressed is the estimated density of the cheese (kg/m^3), x_i and $\rho_i(T)$ are the mass fraction and the density, respectively, of the component *i*: water, fat, protein, carbohydrate, ash and ice (if the temperature is lower than the initial freezing temperature *Tf*; *V(T)* is the estimated volume and *m* is the measured mass of the cheese.

4.3.3 Development of the finite element freezing models

4.3.3.1 Mesh generation

The surface solid of each cheese was meshed using COMSOL Multiphysics™ v. 5.1 (COMSOL Inc., Stockholm, Sweden) into an unstructured tetrahedral mesh type (Figure 3d). The meshing procedure was performed by imposing maximum and minimum element size of $5.25 \cdot 10^{-3}$ and $0.65 \cdot 10^{-3}$ m, respectively, a max element grow ratio of $1.45 \cdot 10^{-3}$ m and curvature factor of 0.5. The parameters were fixed following a mesh-independence analysis that, for simplicity, was evaluated in the case of the fastest cooling rate (C1) (Kumar, Wee, Birla, Subbiah, & Thippareddi, 2012).

4.3.3.2 Thermo-physical properties of the cheese

The initial freezing temperature (T_f) was experimentally determined from the freezing curves by applying the tangent method (Dima et al., 2014).

During the phase change transition of liquid water into ice the thermo-physical properties are strongly dependent on temperature. Therefore, as in the case of density (equation 3), also temperature, composition-dependence of thermal conductivity ($k(T)$) expressed as W/m·K was calculated according to Choi & Okos, (1986) using equations 5:

$$k(T) = \sum_{i=1}^{n} x_i^v k_i(T) \quad (5)$$

where $k_i(T)$ and x^v_i are the thermal conductivity and the volumetric fraction of the component i, respectively.

Components relative fractions were determined by means of compositional near infrared analyses of Mozzarella cheese samples considered; near infrared analyses were performed using a FT-NIR Tango spectrometer (Bruker, MA, USA) according to Alinovi et al., (2019) using commercial calibrations. Ash was calculated by difference.

Temperature-dependence of the ice mass fraction $x_{sw}(T)$ was estimated using the equation proposed by Tchigeov (1979):

$$x_{sw}(T) = x_{tw}\left(\frac{1.105}{1+\frac{0.7138}{\ln(T_f - T+1)}}\right) \quad if\ T < T_f \quad (6)$$

$$x_{sw}(T) = 0 \quad if \quad T > T_f \quad (7)$$

$$x_{lw}(T) = x_{tw} - x_{sw}(T) \quad (8)$$

where $x_{tw}(T)$ and $x_{lw}(T)$, are the mass fractions of total and liquid water fractions of the cheese, respectively. Mass fraction temperature-related variations of x_{sw} and x_{lw} were taken into account into equations 3 and 5.

4.3.3.3 Theoretical model and governing equations

Considering temperature-dependence of thermo-physical properties, the freezing process constitutes a highly non-linear mathematical problem. As the spatial discretization of the mathematical problem implies a 3D geometrical domain, the heat conduction equation of a solid in Cartesian coordinates undergoing phase change transition can be written in the form of Partial Differential Equation (PDE):

$$\rho(T) \cdot Cp(T) \cdot \frac{\partial T}{\partial t} = \nabla \cdot (k(T)\nabla T) \quad (9)$$

According to the apparent specific heat method, equation 10 can be formulated as follows:

$$\rho(T) \cdot Cp_{app}(T) \cdot \frac{\partial T}{\partial t} = \nabla \cdot (k(T)\nabla T) \quad (10)$$

The apparent specific heat $Cp_{app}(T)$ expressed as J/kg·K that has to take into account the amount of latent heat released during the phase change process, was calculated using the series of equations as reported by Voller, Swaminathan, & Thomas, (1990):

$$Cp_{app}(T) = Cp_s(T)x_s + Cp_{lw}(T)x_{lw}(T) + Cp_{sw}(T)x_{sw}(T) + \partial H \frac{dx_{lw}}{dT} \quad (11)$$

with:

$$\partial H = \int_{T_{ref}}^{T} (\rho_{lw} Cp_{lw} - \rho_{sw} Cp_{sw}) dT + \rho_{lw} L \quad (12)$$

where $Cp_s(T)$, $Cp_{lw}(T)$, $Cp_{sw}(T)$ are the specific heat capacity of the dry matter, liquid water and ice fraction of the cheese, respectively; $x_s(T)$, is the mass fractions of the dry matter of the cheese; δH is the difference between liquid and solid (ice) enthalpies; ρ_{lw} and ρ_{sw} are liquid and ice densities, respectively; L is the latent heat of melting of water (333.6 kJ/kg) (Dima et al., 2014; Santos & Lespinard, 2011).

To simulate the convective heat flow during cheese freezing, boundary (14) and initial conditions (13) of the mathematical problem were defined as follows:

$$T = T_0,\ t = 0\ in\ \Omega_c \quad (13)$$

$$-k\left(\frac{\partial T}{\partial x}\cdot n_x + \frac{\partial T}{\partial y}\cdot n_y + \frac{\partial T}{\partial z}\cdot n_z\right) = h(T - T_{ext}), t \geq 0\ in\ \Omega_b \quad (14)$$

where h is the convective heat flow coefficient expressed as W/m^2·K, T_0 (10.7 ± 1.5°C) is the initial temperature of Mozzarella cheese and T_{ext} is the temperature of the freezing chamber, Ω_c is the solid domain of the cheese, Ω_b is the interface between the solid domain of the cheese and the external ambient of the freezing chamber, n_x, n_y, and n_z are the normal outward vector components referred to the system of coordinates. Only the convective contribution to the heat transfer was considered in the whole surface of the cheese domain, as the surface area between the cheese and the HDPE net was negligible.

The governing PDE was spatially discretized into a system of Ordinally Differential Equations (ODE) and solved by applying the Finite Element Method (FEM) using COMSOL Multiphysics™ v. 5.1 and PARDISO direct solver. Time discretization of ODEs was performed by applying the Backward Differentiation Formula (BDF) of order 5 according to Santos, Vampa, Califano, & Zaritzky, (2010) in order to avoid the risk of skipping the latent heat peak at one node during the time stepping process (Delgado & Sun, 2001). Moreover, heat balance error (percentage difference between heat flows through surface and total heat change of product) was calculated in order to monitor accuracy of the models. Freezing models were ran on a Fujitsu Celsius Workstation (Tokyo, Japan) with a 3.5-GHz, 6-cores, Intel Xeon CPU and 32 GB RAM. Time to run the models was about 200 min for models based on photogrammetric geometrical domains and about 75 min for regular geometries.

4.3.3.4 Convective heat transfer coefficient (h) determination

To approximately estimate the heat transfer coefficients (h) of the three different Fc applied during the freezing process of Mozzarella cheeses, the lumped parameter model was applied (Isleroglu & Kaymak-Ertekin, 2016). Three K-type thermocouples connected to a multimeter were positioned in three different locations into the inner part of an aluminium cylinder having similar characteristic dimensions of Mozzarella cheeses (height = 0.046 m, diameter = 0.066 m). The aluminium block was then subjected to the three reported freezing conditions (C1, C2, C3) and time-temperature curves were recorded in triplicate during the cooling process of the object. According to the lumped model, temperatures measured in different locations of the aluminium cylinder were found to be identical.

In order to estimate the *h* coefficients of the three freezing conditions, the following equation was applied to time-temperature curves:

$$-ln\frac{T_{ext}-T(t)}{T_{ext}-T_0}=\frac{h\,SA_s}{(\rho\,Cp\,V)_a}\mathrm{t} \qquad (15)$$

where SA_s, ρ_a, Cp_a, and V_a, were the surface area (0.014 m^2), density (2700 kg/m^3), specific heat capacity (869 J/kg·K) and volume ($1.3\cdot10^{-4}$) of the aluminium cylinder, respectively.

4.3.4 Statistical analysis

A paired t-test was performed using SPSS v.25 (IBM, Armonk, New York) to test if differences between Mozzarella cheese volumes calculated from density values or estimated by the photogrammetric models, and differences between freezing times estimated from different freezing models were significant (P<0.05).

To compare temperature curves of Mozzarella cheeses obtained by computational models and by experimental freezing processes, the roots means square error (RMSE) was calculated as follows:

$$RMSE(°C) = \sqrt{\frac{\sum_{i=1}^{n}\left(\frac{E_i - S_i}{E_i}\right)^2}{n}} \quad (16)$$

where E_i represent the experimental temperature, S_i the simulated temperature obtained from the model, n the number of observations. Moreover, also the maximum absolute deviation (MAD), that calculates the maximum deviation of estimated data from the model and experimental data, was considered:

$$MAD(°C) = max|E_i - S_i| \quad (17)$$

In order to assess the sensitivity of Mozzarella cheese freezing to several process and product parameters, a factorial experimental design based on the Taguchi method was applied (Dar, Meakin, & Aspden, 2002) using Minitab v.17 (State College, Pennsylvania, USA). The parameters considered into the design of experiment, each divided into two levels were: temperature of the freezing chamber (T_{ext}, -34°C, -25°C), h coefficient (36.4 W/m^{2}K, 43.2 W/m^{2}K), *SA:V* ratio of the product (1.035 cm^{-1}, corresponding to a spherical-shape product, 1.121 cm^{-1}, corresponding to a ellipsoidal-shape product), mass of the product (m, 101 g, 102 g) and product's formulation (standard formulation: 63.1 of moisture, 18.3 of proteins, 16.5 of fat, 0.4 of carbohydrates; light formulation: 70.0 of moisture, 18.5 of proteins, 9.0 of fat, 1.0 of carbohydrates). According to the orthogonal design of experiment, eight freezing models were ran to represent a total of 128 (2^8) experiments (Wang & Kolbe, 1994).

4.4 Results and Discussion

4.4.1 Mozzarella cheese thermo-physical properties

Thermo-physical properties (k, ρ, Cp_{app}) were calculated considering the measured mean composition of the cheese (moisture 63.1%, proteins 18.3%, fat 16.5%, lactose and citric acid 0.4%, ashes including NaCl 1.7% w/w), accordingly to equations 3, 5, 11 (**Figure 4a, b, c**). In particular, in **figure 4c** is represented the sharp increase of Cp_{app} that takes into account the latent heat released during the phase change of water into ice and that has to be integrated into the FEM model. Initial freezing temperature (Tf) determined using the tangent method was -0.25°C; this value was higher from the data by Ribero, Rubiolo, & Zorrilla (2007) that observed freezing points between -1.2°C and -2.4°C. This difference is related to the moisture content of the samples, significantly higher than the one reported by Ribero et al. (2007) (50.6%) and presumably by a lower concentration of solutes that affects colligative properties such as the freezing point depression.

4.4.2 Convective heat transfer coefficient of the processes

The h coefficient of three different applied Fc determined according to equation 15, were 43.2 ± 0.7 W/m^{2}K for C1 (-40°C, 4.1 ± 0.6 m/s), 39.5 ± 0.6 W/m^{2}K for C2 (-31°C, 2.5 ± 0.4 m/s) and 36.4 ± 0.5 W/m^{2}K for C3 (-25°C, 1.3 ± 0.2 m/s). Results showed the expected increase of the surface heat transfer with the lowering of the air temperature and the increasing air velocity into the freezing chamber, in accordance with Ghisalberti & Kondjoyan (1999).

4.4.3 Photogrammetric reconstruction of Mozzarella cheese spatial domain

Results from the photogrammetric reconstruction of Mozzarella cheese samples were in good accordance with the calculated volumes by using equation 3 and 4. Differences between the reported sample volumes were equal or lower than 2%, with an average difference of -0.6% (**Table 2)**. Zhang et al. (2016) reported a lower relative error (<1%) of photogrammetric results compared to the reference volumes of eggs, but on the contrary they observed a consistent bias caused by the mesh approximation of the photogrammetric volume. In our study, there was no significant bias between reference and photogrammetric measurements (P = 0.269).

The different shape and dimensions influenced the surface area-to-volume ratio of each cheese sample (*SA:V*) (Table 2). Moreover, mean *SA:V* (1.121 ± 0.025 cm^{-1}) was strongly different from the surface area-to-volume ratio of regular shape solids, calculated using equations 18 and 19:

$$SA{:}V_{sphere} = {}^{3}/_{r} \tag{18}$$

$$SA{:}V_{ellipsoid} = \frac{3}{abc}\sqrt[p]{\frac{a^p b^p + a^p c^p + b^p c^p}{3}} \tag{19}$$

where *r* is the radius of the sphere, *a, b, c* are the semi-axes of the ellipsoid and *p* was approximated to 1.6075 (Carbognani et al., 2012). By imposing *r* = 2.884 cm, *a* =*b* = 3.100 cm, *c* = 2.497 cm, that corresponded to solid geometries with an approximated volume of 100.5 cm^3, *SA:V* were 1.040 cm^{-1} and 1.049 cm^{-1} in the case of a sphere and oblate ellipsoid, respectively (supplementary material, Table 1).

To develop a simplified ellipsoid domain characterized by the average *SA:V* of Mozzarella cheese samples measured with the photogrammetric technique, the following system of equations can be solved:

$$\begin{cases} SA_{ellipsoid} = 4\pi\sqrt[p]{\frac{a^p b^p + a^p c^p + b^p c^p}{3}} \\ V_{ellipsoid} = \frac{4}{3}\pi\, abc \end{cases} \tag{20, 21}$$

where $SA_{ellipsoid}$ is the surface area of the ellipsoid and $V_{ellipsoid}$ is the volume. For example, by imposing *a* > *b* = *c*, $SA_{ellipsoid}$ = 114.29 cm^2, that is the mean *SA* of cheese samples, and $V_{ellipsoid}$ = 100.5 cm^3, that is the *V* value calculated using equation 4 considering the nominal mass of Mozzarella cheese (100.0 g), it is possible to create a prolate ellipsoid having *a* = 4.833 cm and *b* = *c* = 2.228 cm, characterized by the measured mean *SA:V* value. This procedure was also used to create geometries based on the measured mass of each sample in order to create freezing models based on a simplified prolate ellipsoidal domains but at the same time on the estimated average *SA:V* of the real samples (supplementary material, Table 1).

4.4.4 Validation of the freezing models based on the photogrammetric domain of the cheese

In **Figure 5** are reported as example the comparison between the experimental data and the predicted temperature curves from the freezing models of three Mozzarella cheese samples (C1 a, C2 a, C3 a). In order to validate the models, the position of the thermocouples in the external and in the central part of the cheese was measured during the experimental trials using a caliper and was tracked during the photogrammetric procedure and FEM post-processing (in terms of x, y, z coordinates of the photogrammetric model), in order to compare predicted with experimental results. Experimental and boundary conditions were those reported in paragraph 2.1, while *h* coefficients were those calculated in paragraph 3.2.

As it is possible to observe from the graphs, there was a good agreement between experimental and predicted data during the whole freezing process and for all the considered freezing conditions. This result was confirmed by the low values of RMSE (lower than 1.47°C) and MAD (lower than 3.4°C, with the exception of sample C2 a, that was 4.17°C) (supplementary material, **Table 3)**. Also the highest MAD value did not negatively influence the freezing time prediction of the sample, that was still accurate (**Figure 5b**). Freezing models based on the photogrammetric reconstruction of Mozzarella cheese can be considered validated and can be used to predict the freezing time and the temperature profiles in different locations of the cheese with a high degree of accuracy (Dima et al., 2014).

4.4.5 Effect of cheese geometry on the freezing process: comparison between photogrammetric and simple-geometry models

In order to demonstrate the importance of applying the photogrammetric reconstruction of Mozzarella cheese spatial domain, freezing models based on simple geometries (sphere, ellipsoids) were built, as previously discussed in paragraph 2.2.2.

Freezing models were built considering T_0=11°C and a final freezing temperature of -20°C. As the experimental conditions were set up in order to have homogeneous boundary conditions on the surface of Mozzarella cheese, the warmest point during the freezing processes was observed in a fixed position corresponding to the geometrical center (also equal to the center of mass at t_0, considering $\rho(T_0)$ constant in all the points of the cheese domain) of the non-regular shape solid with coordinates (x=0, y=0, z=0) (Lespinard et al., 2009).

Models built considering the nominal cheese mass of the cheese (100.0 g) gave only slight differences (**Figure 6)** if compared with models based on the effective, measured mass of the samples ranging from 99.8 to 102.0 g (**Table 2**). On the contrary, as it is possible to observe for all the three freezing conditions, models based on spheres or oblate ellipsoids gave always different temperature profiles than those based on photogrammetric geometries (**Figure 6)**. Moreover, these models predicted significantly ($P<0.05$) longer freezing times to reach -20°C in the geometrical center of the cheese (about 3 to 5 min) (supplementary material, **Table 4**). This difference can be attributed to the non-homogeneous heat flux generated through Mozzarella cheese with non-regular geometry (photogrammetric domain) during the freezing process, as the distance from the center to the surface of the cheese was not constant. The non-homogeneous heat flux through the cheese body caused also the formation of evident temperature-surface differences (up to 8-9°C) (**Figure 7**). Local temperature differences can be responsible for cheese defects, as surface cracking (Reid & Yan, 2004). The temperature-planar distribution (zy, xy planes) predictable during the freezing process showed that temperature differences follow the non-regular shape of the cheese (**Figure 8**). These non-homogeneities were enhanced at higher freezing rates, and can negatively affect product's characteristics.

On the contrary, models based on prolate ellipsoids that were characterized by spatial domains with similar *SA:V* values if compared with the photogrammetric domains, showed similar freezing times ($P=0.531$, average difference of 0.4°C) and temperature curves in the center of the cheese to the photogrammetric models (supplementary material, **Table 4**).

These results show that the *SA:V* value of the cheese estimated by photogrammetry, is an important, critical parameter that can be related to the time-to-freeze of a non-regular shaped product such as Mozzarella cheese. Modelling of Mozzarella cheese freezing process can be performed by building simplified, regular geometries according to the estimated *SA:V* that can be useful to predict the freezing time in a simple way, without considering temperature distribution in the outer part of the cheese, or by directly using the photogrammetric domain.

4.4.6 Effect of process conditions, product's geometry, mass and composition on Mozzarella cheese freezing times

3.1 freezing times

The main effect of different process parameters (T_{ext}, *h* coefficient) and product's characteristics (*SA:V*, *m*, product's formulation) on Mozzarella cheese freezing time (min) to reach -20°C can be observed in **Figure 9**.

Amongst all, T_{ext} showed the strongest influence on Mozzarella cheese freezing time; this parameter led to a mean freezing time of 24.7 min longer at -25°C than at -34°C. The other important parameters in defining the freezing time of the cheese were, in order of importance, *SA:V* and *h*, that resulted in a mean difference of 7.5 and 6.0 min, respectively, between level 1 and 2. This observation confirmed the importance of controlling and measuring the *SA:V* of the product, in order to obtain an accurate prediction of the freezing time and to rationally perform the process in relation with product's shape and dimension. For example, an ellipsoidal cheese shape with higher *SA:V* than that of a sphere, can fasten the freezing process and consequently could reduce energetical costs; on the other hand, the process would be also affected by higher temperature non-homogeneities in the outer parts of the cheese that, if not controlled, they could cause product's defects such as skin's peeling off or cracking phenomena (Laurienzo et al., 2008; Reid & Yan, 2004). Finally, *m* and product's formulation had the lower impact on freezing time. In particular, small variations of estimated cheese mass (± 1g), that was of the same extent to the mass variability of Mozzarella cheese encountered considering a larger sample size (n=200 during a well performed shaping process, have a negligible effect over product freezing time, in accordance to **Figure 6**.

4.5 Conclusions

HM Mozzarella cheese is characterized by a non-regular shape that influences the freezing time and the homogeneity of temperature distribution.

Modelling of HM Mozzarella cheese freezing process by coupling the finite element method and a photogrammetric procedure allowed to more accurately predict the freezing process than using regular geometries.

The photogrammetric procedure allowed to calculate the average *SA:V* value of the cheeses, and to build a simplified prolate ellipsoidal geometry that was useful to predict the freezing time of the cheese with good results. Freezing models highlighted that the surface area-to-volume ratio of the product is one of the most critical parameters that define the freezing time of cheese and has to be take into account when the process has to be optimized in industrial applications.

Factors affecting the shape variability of HM Mozzarella cheese intended to be frozen should be better controlled by dairies to improve the reproducibility of the process and cheese quality.

The coupled photogrammetric - finite element method modelling approach can be implemented into computational fluid dynamic studies to evaluate the convective flows and temperature profiles into freezing tunnels or chambers and, in general, can be used to build three-dimensional models of heat exchange or mass transfer of different kind of non-regular shape products.

4.6 Conflicts of Interest

The authors declare no potential conflicts of interest concerning the research, authorship, and/or publication of this article.

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4.8 References

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4.9 Figures and tables

Figure 1. Schematic representation of the experimental setup used to perform the freezing experiments of Mozzarella cheeses.

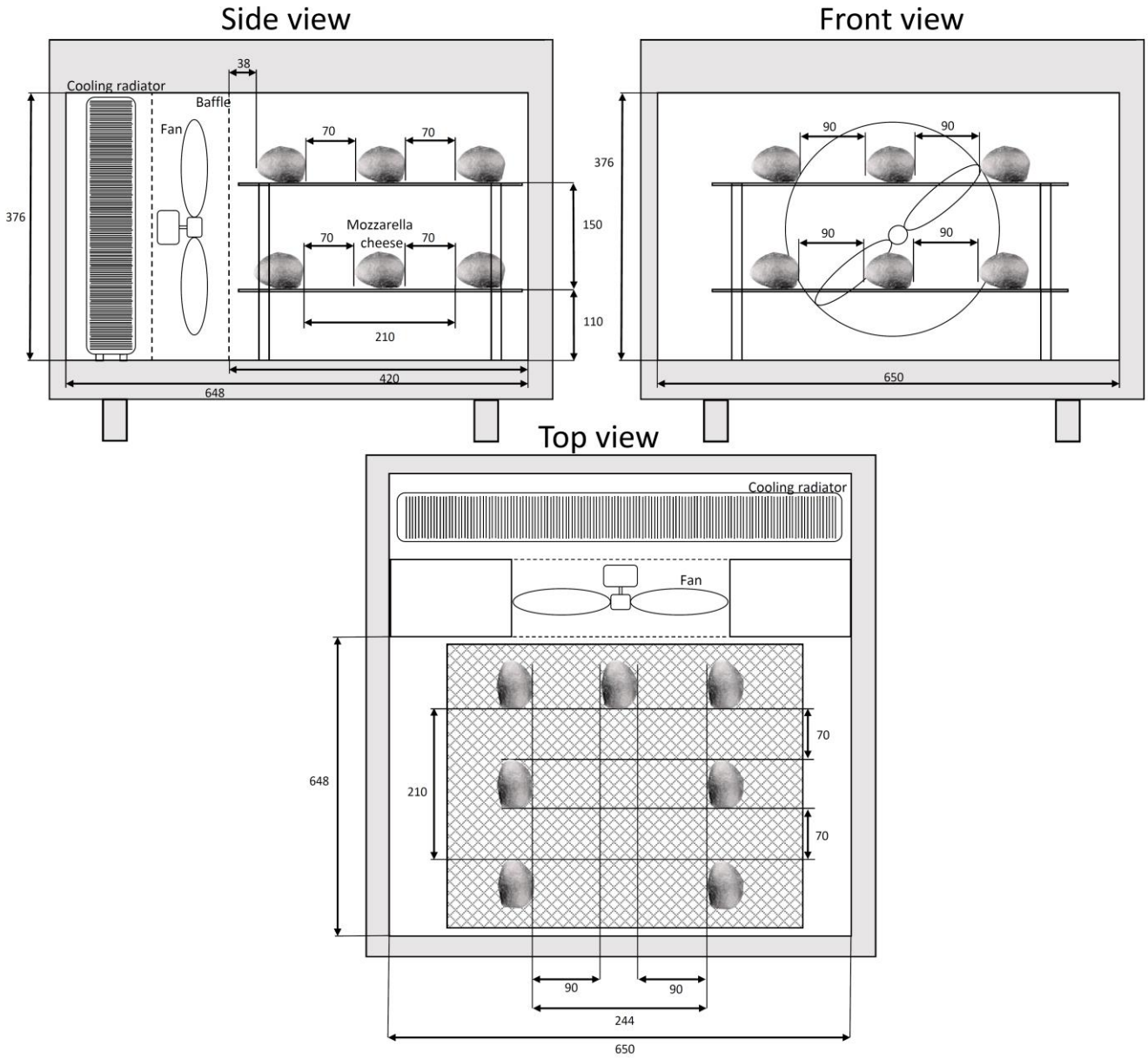

Figure 2. Representation of the photogrammetric setup and equipment used to reconstruct the geometrical domain of Mozzarella cheese samples.

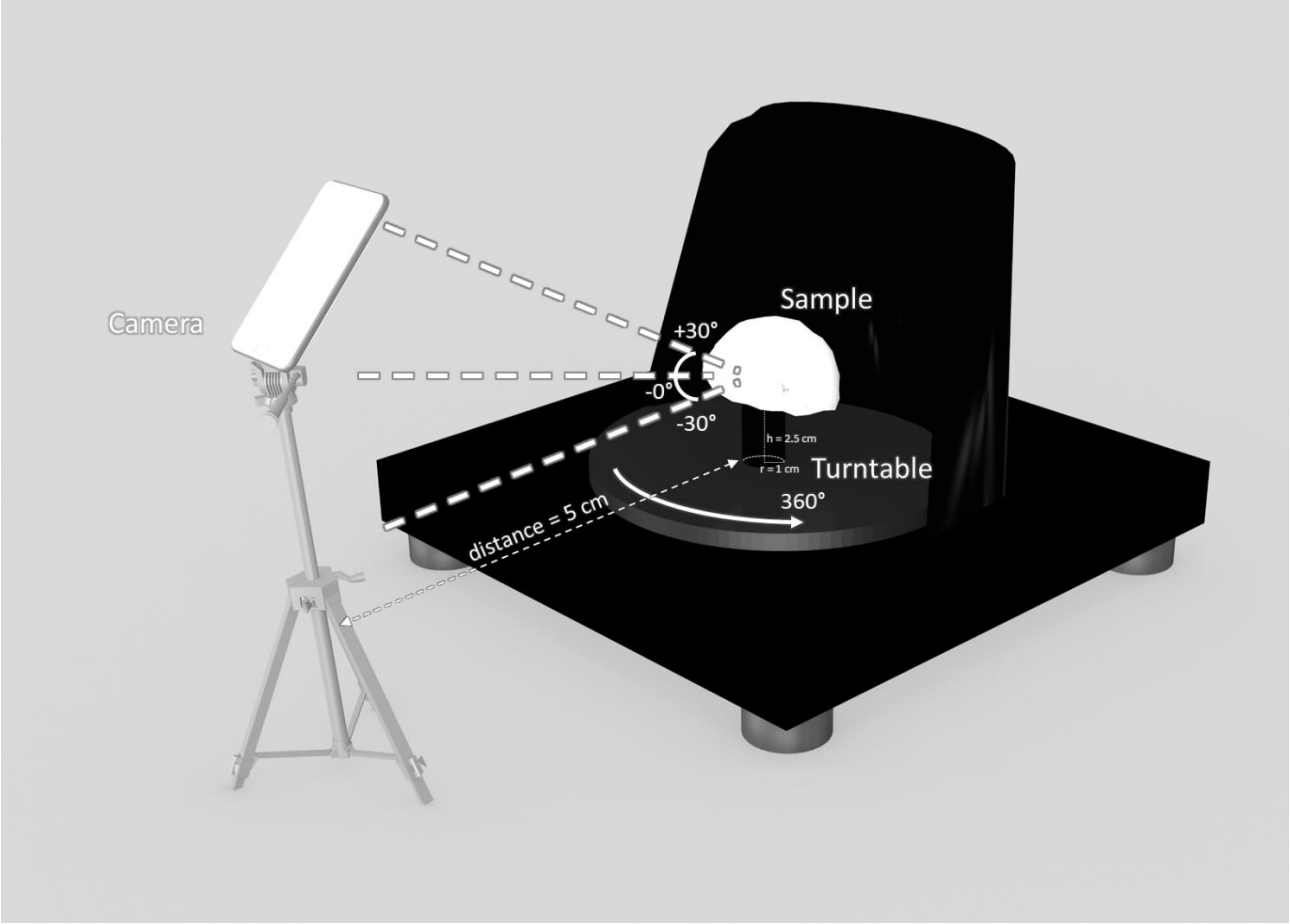

Figure 3. Mozzarella cheese geometry reconstruction and meshing: (a) photo acquisition, (b) texturized surface of the solid constructed from high accuracy dense point cloud (PhotoScan v. 1.2, Agisoft, St. Petersburg, Russia), (c) simplified surface solid domain (Solidworks 2017, Dassault Systèmes, Vélizy Villacoublay, France), (d) meshed surface solid (COMSOL Multiphysics™ v. 5.1 (COMSOL Inc., Stockholm, Sweden), (e) developed photogrammetry workflow.

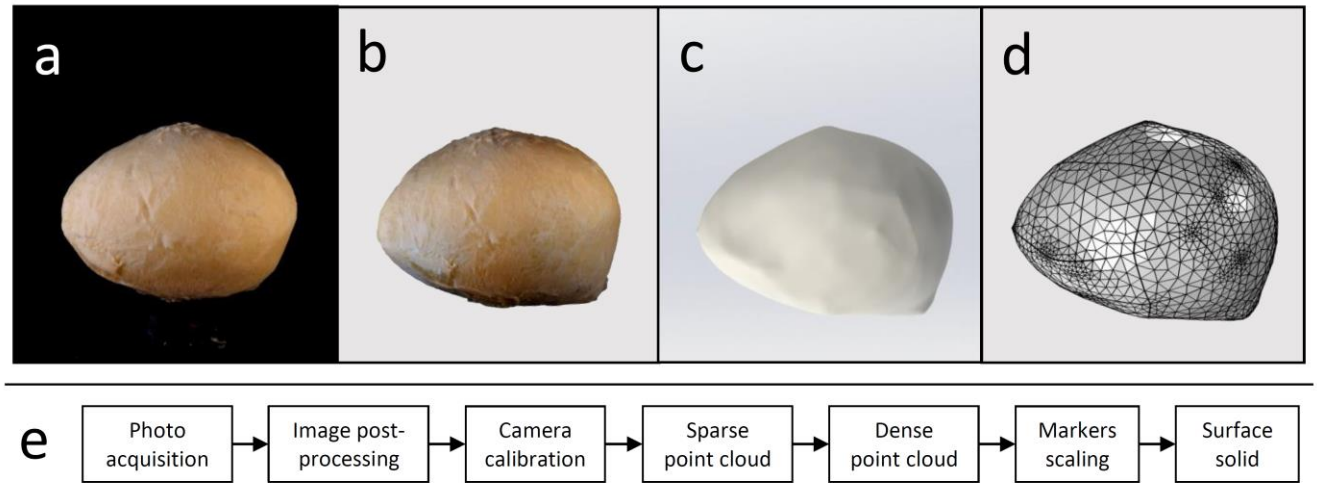

Figure 4. Thermo-physical properties of Mozzarella cheese expressed as a function of temperature: (a) thermal conductivity, (b) density, (c) apparent specific heat, (d) mass fraction of ice.

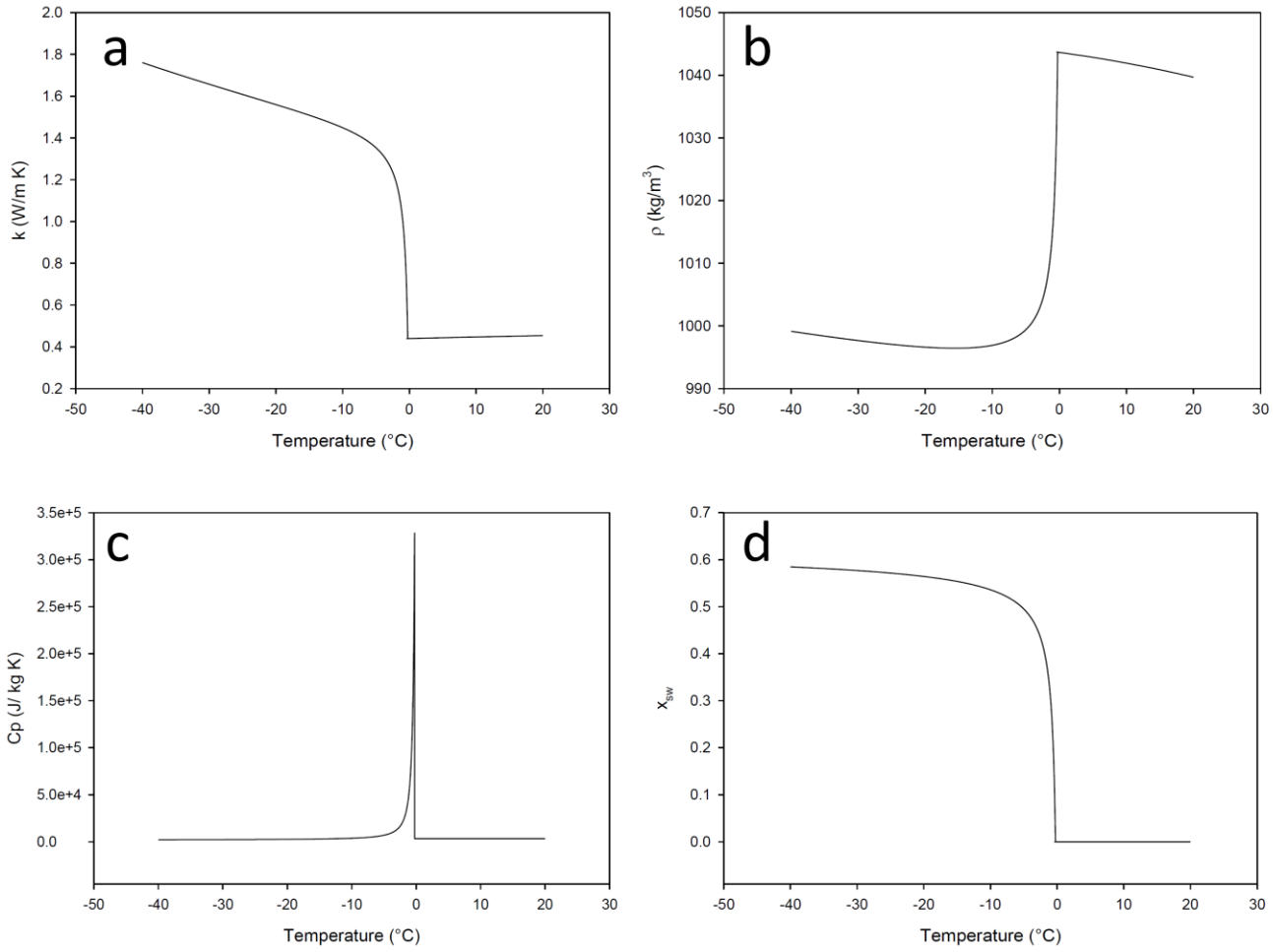

Figure 5. Comparison between experimental and estimated temperature curves from the freezing models of Mozzarella cheese of C1 a, C2 a, C3 a sample. Dots represent temperatures estimated from the models: (○) surface, (●) central point of the cheese; lines are experimental data (_ _ _) surface, (_____) central point of the cheese. (a) sample C1 a: -34.0 ± 2.4°C, 4.1 ± 0.6 m/s, (b) sample C2 a: -30.0 ± 2.4°C, 2.5 ± 0.4 m/s, (c) sample C3 a: -24.4 ± 1.5°C, 1.3 ± 0.2 m/s.

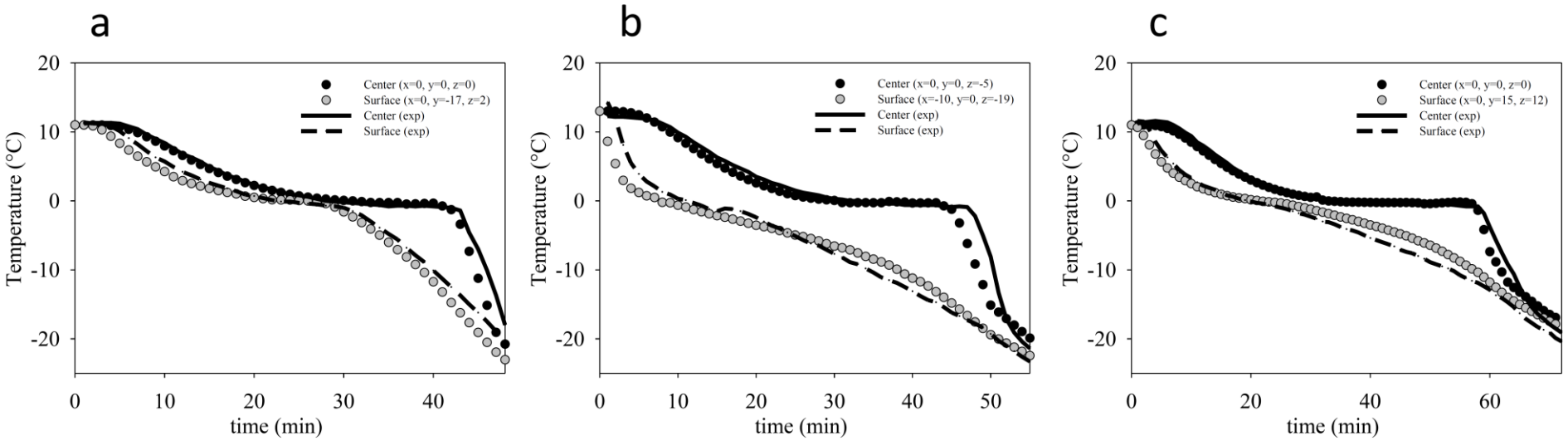

Figure 6. Comparison of temperature curves obtained from freezing models of Mozzarella cheese based on photogrammetric and simple spatial domains (C1 a, C2 a, C3 a sample). (●) photogrammetric domain of the cheese, (○) 100.0 g sphere, (▼) sphere built from the real (measured) weight of the sample, (▲) 100.0 g oblate ellipsoid, (■) oblate ellipsoid built from the real (measured) weight of the sample, (□) 100.0 g prolate ellipsoid, (◆)prolate ellipsoid built from the real (measured) weight of the sample. (a) sample C1 a: -34.0 ± 2.4°C, 4.1 ± 0.6 m/s, (b) sample C2 a: -30.0 ± 2.4°C, 2.5 ± 0.4 m/s, (c) sample C3 a: -24.4 ± 1.5°C, 1.3 ± 0.2 m/s.

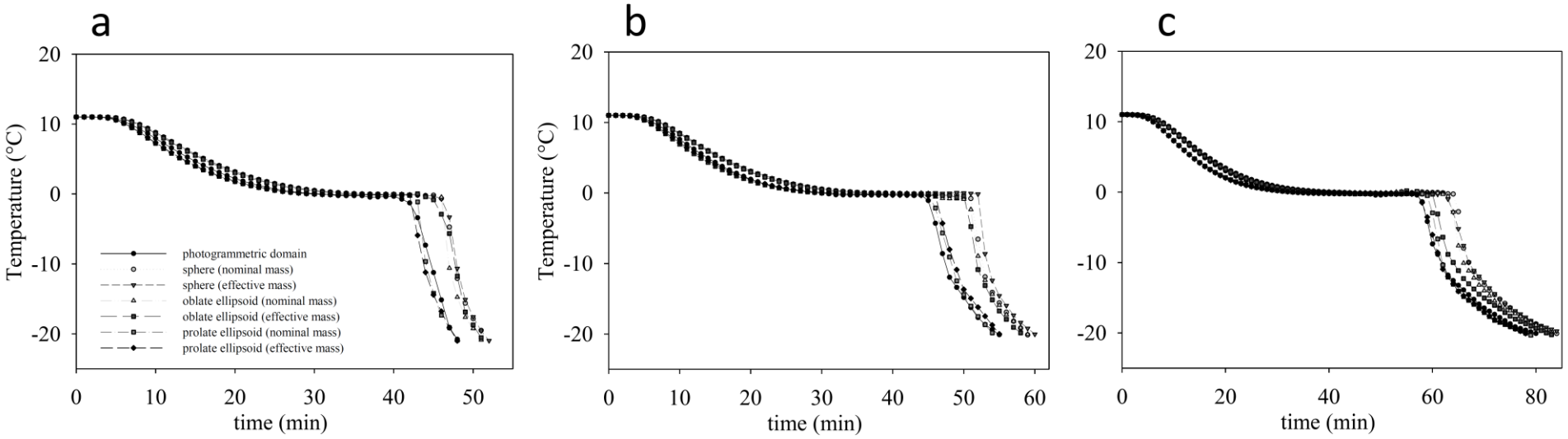

Figure 7. Temperature-surface differences during the freezing process of Mozzarella cheese samples C1 a (a, b, c), C2 a (d, e, f), C3 a (g, h, i), at three process times: 5 min (a, d, g), 20 min (b, e, h), 40 min (c, f, i); sample C1 a: -34.0 ± 2.4°C, 4.1 ± 0.6 m/s, sample C2 a: -30.0 ± 2.4°C, 2.5 ± 0.4 m/s, sample C3 a: -24.4 ± 1.5°C, 1.3 ± 0.2 m/s.

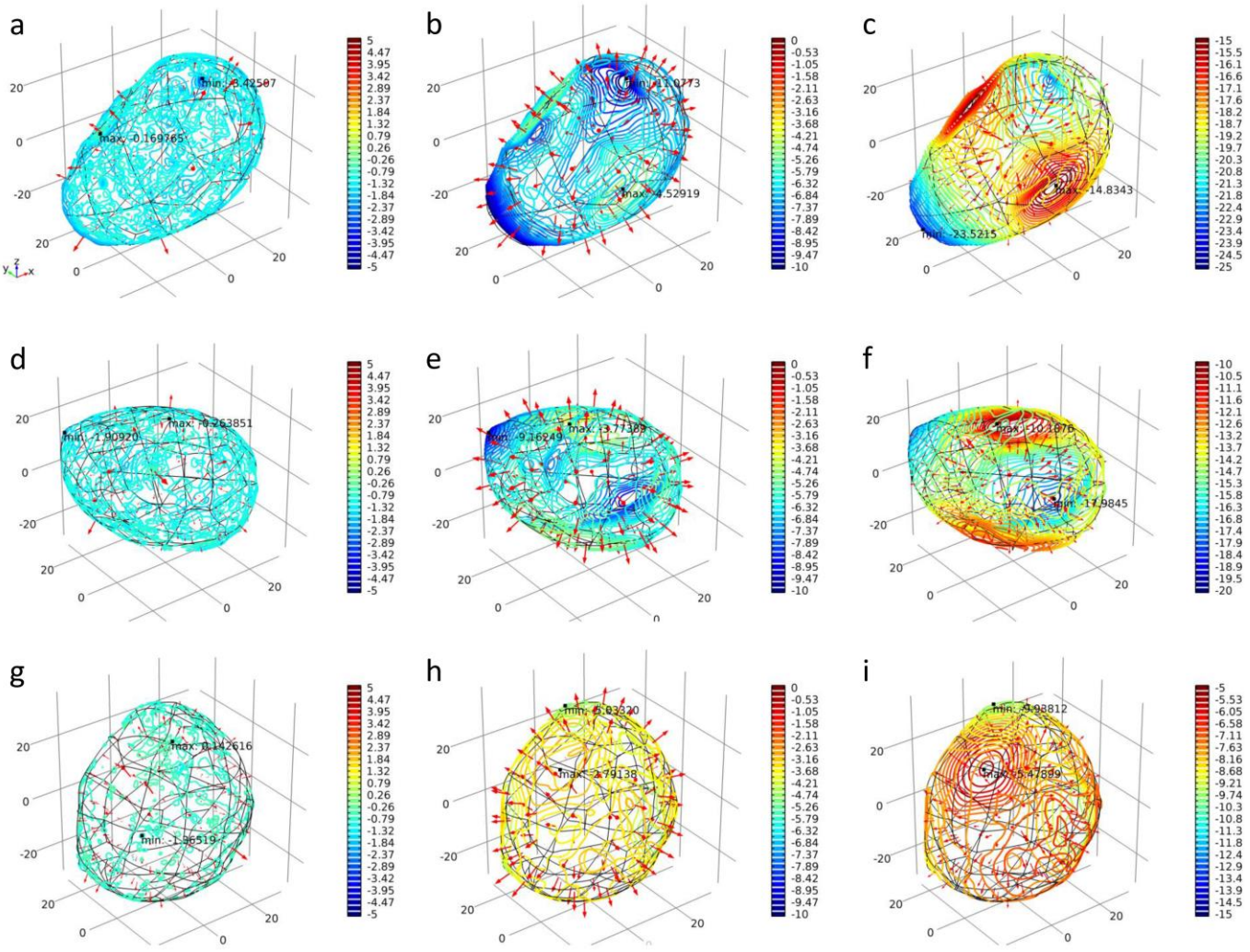

Figure 8. Temperature-planar distribution (zy, xy planes) during the freezing process of Mozzarella cheese samples C1 a (a, b, c), C2 a (d, e, f), C3 a (g, h, i), at three process times: 5 min (a, d, g), 20 min (b, e, h), 40 min (c, f, i); sample C1 a: -34.0 ± 2.4°C, 4.1 ± 0.6 m/s, sample C2 a: -30.0 ± 2.4°C, 2.5 ± 0.4 m/s, sample C3 a: -24.4 ± 1.5°C, 1.3 ± 0.2 m/s.

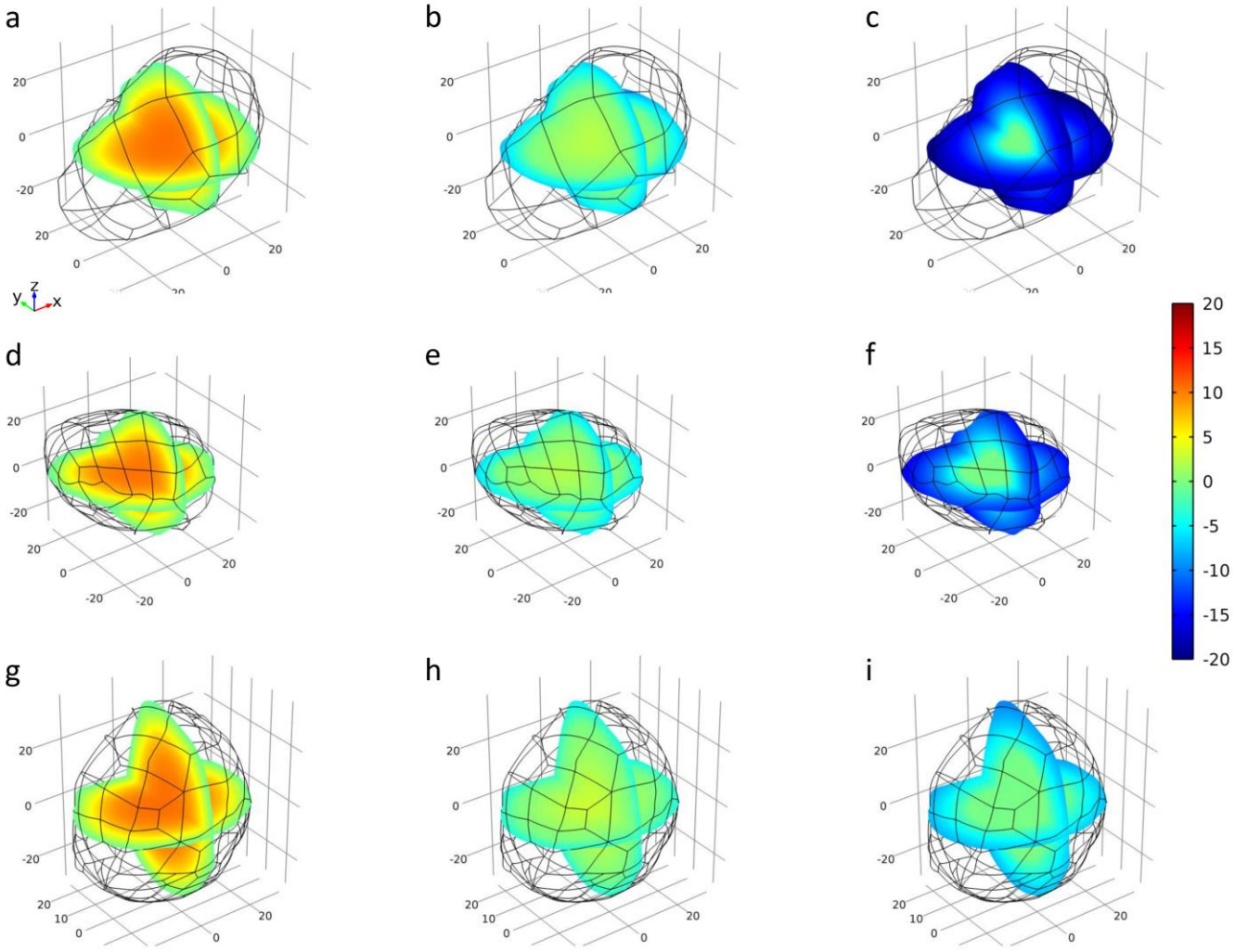

Figure 9. Graphical representation of process and product's parameters on the Mozzarella cheese freezing time (min). The dashed line represents the overall experimental mean. Considered factors and levels were: temperature of the freezing chamber (T_{ext}, -34°C, -25°C), *h* coefficient (36.4 W/m^{2}K, 43.2 W/m^{2}K), *SA:V* of the product (1.035 cm^{-1}, 1.121 cm^{-1}), cheese mass (101 g, 102 g) and product's formulation (standard formulation: 63.1 of moisture, 18.3 of proteins, 16.5 of fat, 0.4 of carbohydrates; light formulation: 70.0 of moisture, 18.5 of proteins, 9.0 of fat, 1.0 of carbohydrates).

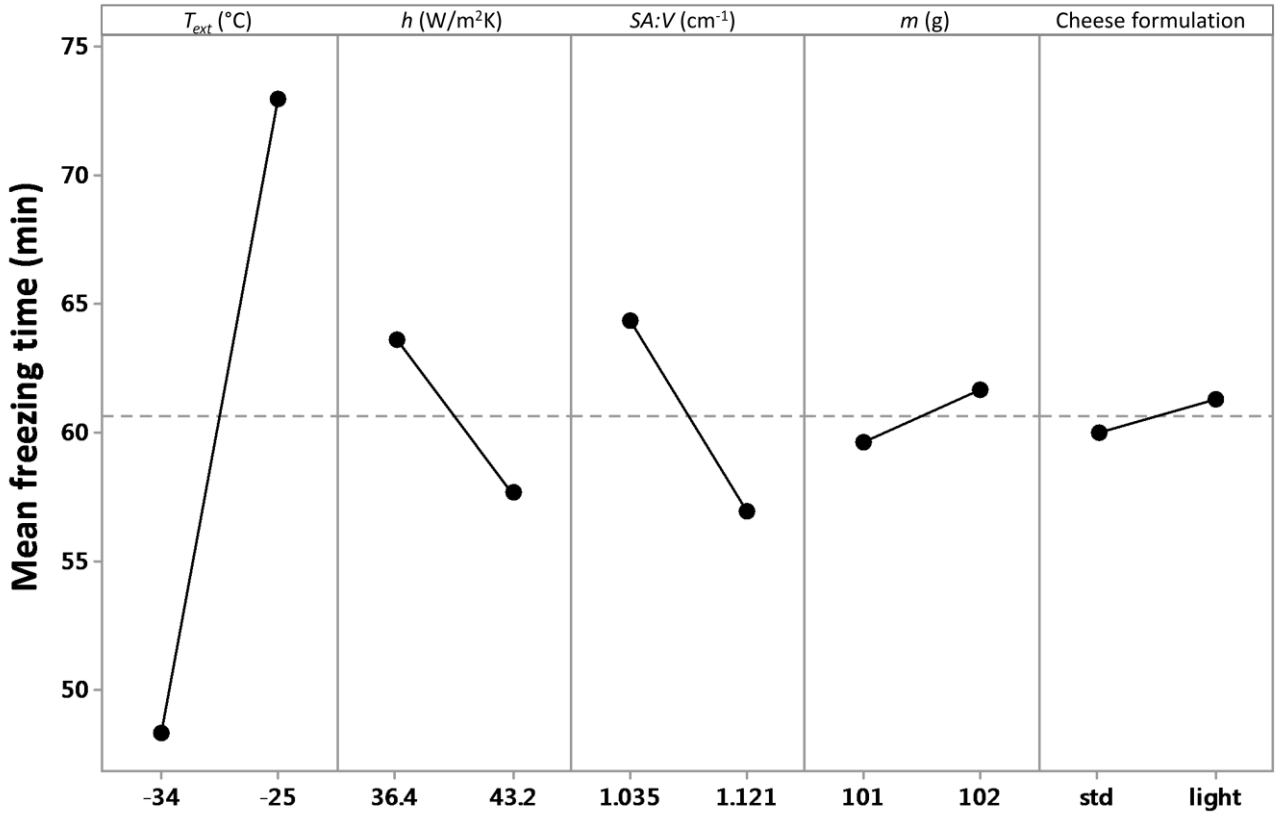

Table 1. Characteristics of spatial domains based on regular geometries (sphere, oblate and prolate ellipsoid) used to model the freezing process of Mozzarella cheese; *r* is the radius of the sphere, *a, b, c* are the semi-axes of the ellipsoids, *m* is the measured mass of the cheese, *V* is the calculated volume of the solid using equation 4, *SA:V* is the surface area-to-volume ratio.

			Sphere		Oblate ellipsoid				Prolate ellipsoid			
Sample	*m* (g)	*V* (cm^3)*	*r* (cm)	*SA:V* (cm^{-1})	*a* (cm)	*b* (cm)	*c* (cm)	*SA:V* (cm^{-1})	*a* (cm)	*b* (cm)	*c* (cm)	*SA:V* (cm^{-1})
Nominal weight	100.0	100.5	2.884	1.040	3.100	3.100	2.497	1.049	4.833	2.228	2.228	1.138
C1 a	100.7	101.2	2.891	1.038	3.106	3.106	2.505	1.047	4.771	2.251	2.251	1.130
C1 b	100.5	101.0	2.889	1.038	3.105	3.105	2.503	1.047	4.788	2.2441	2.244	1.132
C2 a	101.3	101.8	2.897	1.036	3.113	3.113	2.510	1.044	4.718	2.270	2.270	1.123
C2 b	102.0	102.5	2.903	1.033	3.120	3.120	2.515	1.042	4.656	2.293	2.293	1.115
C3 a	101.0	101.5	2.894	1.037	3.110	3.110	2.508	1.045	4.744	2.260	2.260	1.126
C3 b	99.8	99.7	2.835	1.058	3.097	3.097	2.497	1.050	4.850	2.222	2.222	1.140

*calculated using $\rho(-20°C)$ = 0.995 kg/m^3

Table 2. Comparison between volumes of Mozzarella cheese samples estimated using the photogrammetric technique and calculated from measured sample mass and surface area-to-volume ratio (*SA:V*) of the cheeses, expressed as cm^{-1}; *m* (g) is the measured mass of the cheese; m_{photo} (g) is the calculated mass of the cheese from photogrammetric model; *V* (cm^3) is the calculated volume of the solid using equation 4; V_{photo} (cm^3) is the estimated volume from photogrammetric model; *SA* (cm^2) is the estimated surface area from the photogrammetric model.

Sample	*m* (g)	m_{photo} (g)*	*V* (cm^3)*	V_{photo} (cm^3)	*SA* (cm^2)	Difference (V-V_{photo}) (%)	*SA:V* (cm^{-1})
C1 a	100.7	100.4	101.2	100.9	115.5	0.3	1.14
C1 b	100.5	102.6	101.0	103.0	114.0	-2.0	1.11
C2 a	101.3	102.2	101.8	102.7	118.7	-0.9	1.15
C2 b	102.0	102.2	102.5	102.8	115.0	-0.2	1.12
C3 a	101.0	99.9	101.5	100.4	109.1	1.1	1.09
C3 b	99.2	101.2	99.7	101.7	113.4	-2.0	1.11

*calculated using $\rho(-20°C)$ = 0.995 kg/m^3

Table 3. Root Mean Square Error (RMSE) and Maximum Absolute Difference (MAD) between experimental and numerical temperature curves of Mozzarella cheese freezing processes (C1: -34.0 ± 2.4°C, 4.1 ± 0.6 m/s, C2: -30.0 ± 2.4°C, 2.5 ± 0.4 m/s, C3: -24.4 ± 1.5°C, 1.3 ± 0.2 m/s). Freezing models were developed by applying the photogrammetric procedure to reconstruct the spatial domain of the cheese.

Freezing condition	Sample	Coordinates (mm)	RMSE (°C)	MAD (°C)
C1	a	central part (x=0, y=0, z=-2)	0.08	1.51
		outer part (x=0, y=-18, z=2)	0.35	1.40
	b	central part (x=0, y=0, z=0)	0.12	1.46
		outer part (x=0, y=-17, z=2)	0.25	1.65
C2	a	central part (x=0, y=0, z=-5)	0.78	4.17
		outer part (x=-10, y=0, z=-19)	1.39	3.37
	b	central part (x=0, y=0, z=-4)	0.53	3.06
		outer part (x=-10, y=1, z=-18)	0.31	1.39
C3	a	central part (x=0, y=0, z=0)	0.26	2.17
		outer part (x=0, y=15, z=12)	1.44	2.79
	b	central part (x=0, y=-18, z=5)	0.41	2.73
		outer part (x=-6, y=2, z=-22)	1.47	2.80

Table 4. Comparison among Mozzarella freezing models based on photogrammetric and simple geometries domains of the cheese (sphere, oblate ellipsoid and prolate ellipsoid) built considering the real mass of the cheeses: freezing times to reach -20°C in the core of the product and Root Mean Squared Errors (RMSE) of temperature curves.

		Photogrammetric geometry	Sphere		Oblate ellipsoid		Prolate ellipsoid	
Fc	Sample	$t_{-20°C}$	$t_{-20°C}$ (min)	RMSE (°C)	$t_{-20°C}$ (min)	RMSE (°C)	$t_{-20°C}$ (min)	RMSE (°C)
C1	a	48.6	52.4	27.59	51.6	24.37	48.5	6.41
	b	50.5	55.2	35.67	54.2	29.67	51.1	6.81
C2	a	55.9	60.9	37.87	59.2	29.25	56.2	8.11
	b	54.2	59.2	27.30	59.1	31.57	55.5	13.97
C3	a	80.7	86.0	26.23	83.1	12.64	78.8	5.59
	b	80.1	84.7	25.14	83.9	14.73	77.3	9.33

5. Effect of freezing and thawing processes on high-moisture Mozzarella cheese rheological and physical properties

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5.1 Abstract

High-moisture Mozzarella cheese is a soft, fresh cheese characterized by a short shelf-life, but a freezing process can be effective for improving its storability. In this study, the effects of two freezing/thawing methods (the presence or absence of a covering liquid during the process), three freezing (ranging from -40 °C, 4.1 ± 0.6 m/s, to -25 °C, 1.3 ± 0.2 m/s) and two thawing conditions (+4 °C, 1.3 ± 0.2 m/s, +4 °C, 4.1 ± 0.6 m/s) were evaluated on Mozzarella cheese characteristics. Cheeses with a covering liquid were characterized by water absorption during thawing, lower water holding capacity, softer texture and lower rheological moduli. Frozen/thawed cheeses without the covering liquid and stored overnight with a new covering liquid, despite having a lower juiciness, were characterized by a lower degree of freezing-induced modifications and were more similar to the fresh cheese. Cheese properties were not largely affected by the freezing/thawing conditions considered here. Freezing high-moisture Mozzarella cheese has a small impact on a product's properties if it is performed without a covering liquid and is followed by an overnight rehydration step in a fresh covering liquid. Therefore, this should be the preferred method to obtain the best quality results.

Keywords: Mozzarella cheese; Cheese texture; Sensory analysis; Expressible serum; Air-blast freezing

5.2 Introduction

Mozzarella cheese, one of the most consumed cheese worldwide, can be divided into two categories related to its final utilization: for consumption as a fresh cheese or as an ingredient for pizza or other prepared foods (Francolino, Locci, Ghiglietti, Iezzi, & Mucchetti, 2010). Functional characteristics, such as the shreddability and meltability, largely depend on the cheese moisture content (Bertola, Califano, Bevilacqua, & Zaritzky, 1996), and they are poor in the case of high-moisture (HM) Mozzarella cheese, which has a moisture content between 52 and 60 g/100 g (U.S. FDA, 2018) or higher in the case of the Italian-type. Thus, HM Mozzarella cheese is preferably consumed as a fresh cheese and is packed and stored with a covering liquid that is a brine containing mono, divalent salts and/or organic acids (NaCl, CaCl2, Calcium lactate) (Faccia, Angiolillo, Mastromatteo, Conte, & Del Nobile, 2013). This liquid is useful to maintain the high moisture of the cheese, to avoid the formation of a rind and eventually to complete cheese salting. Because of its high moisture content and the fresh taste expected by the consumer, HM Mozzarella cheese is characterized by a short shelf-life that can vary from one to thirty days (Mucchetti, Pugliese, & Paciulli, 2016).

To improve the storability, freezing has been assessed in the last few years for several cheeses (Alberini, Miccolo, & Rubiolo, 2015; Alvarenga, Canada, & Sousa, 2011; Conte et al., 2017; Reid & Yan, 2004; Kuo, Anderson, & Gunasekaran, 2003). Considering the globalization of food markets and the increasing demand for highly perishable cheeses, freezing can be a good strategy to decrease waste by improving, for example, a product's convenience and supply chain efficiency (Pollack, 2001).

Patents concerning HM Mozzarella cheese or curd freezing have been published (Coker, Gillies, Havea, & Taylor, 2017; Zambrini & Bernardi, 2017), and the process is currently performed on an industrial scale, despite HM Mozzarella cheese not being considered suitable for freezing in the US (USDEC, 2016). However, there is a lack of scientific data about the effects of freezing and thawing on HM Mozzarella cheese characteristics. HM Mozzarella cheese can be frozen by an individual quick freezing (IQF) method as a packaged product immersed in its covering liquid or as a non-packaged product.

Conte et al., (2017) compared the effects of freezing rate and 2-months of frozen storage on "fiordilatte" Mozzarella cheeses. Higher freezing rates preserved better cheese quality. However, the authors measured a decreased pores volume and overall sensory quality probably caused by the increase in firmness that can be related to ice formation and subsequent protein dehydration, as reported by other authors for different cheeses (Alvarenga et al., 2011; Diefes, Rizvi, & Bartsch, 1993;

Reid & Yan, 2004). Considering its high moisture content, HM Mozzarella cheese can be sensitive to freezing, thus, the process must be closely controlled and tailored in terms of the freezing rate and methods (Alvarenga, Ferro, Almodôvar, Canada, & Sousa, 2013).

In this context, the objective of this work was to evaluate the effects of different freezing/thawing methods and processing conditions over HM Mozzarella cheese characteristics to find the best process parameters that can lead to optimal quality results.

5.3 Material and methods

5.3.1 Experimental design

Four 100 g batches of fresh HM Mozzarella cheese were kindly provided by Alival S.p.a. (Nuova Castelli S.p.a. RE, Italy). The cheeses used for the study were produced on different days in a two-months period. Cheeses were manufactured using standard cow milk (3.30 g/100 g protein, 3.50 g/100 g fat) pasteurized at 74 °C for 25 s; 1.2 g/100 g of citric acid and Microbial rennet were added to start milk coagulation. After cheese curd stretching with salted boiling water, cheeses were moulded into 100 g individual spheroidal shapes and cooled by immersion in tap water. Each product's final gross composition was 17.0 g of protein, 17.0 g of fat, 1.0 g of lactose and 0.4 g of NaCl. Cheeses were individually packaged into polyethylene bags containing 100 g of covering liquid (0.4 g/100 g NaCl) and then were kept at 4 ± 1 °C for 5 d before being frozen. For each manufacturing batch, 91 cheeses were considered (**Figure 1**).

Samples were frozen by applying two freezing/thawing methods (***M***). Cheeses were frozen with (***M_w***) or without (***M_d***) covering liquid using an air blast freezer (MF 25.1, Irinox, TV, Italy) until a temperature of -20 °C in the core of the product was reached. M_d-treatments were separated from the covering liquid before freezing, while M_w-treatments were unpackaged and poured into truncated cone-shaped polypropylene containers (r_1 = 10 cm r_2 = 8 cm, height = 9 cm) with their original covering liquid.

Three freezing conditions (***Fc***), governed by the air temperature and velocity in the freezing chamber, were applied, as reported in **Figure 1**. Cheeses were stored at -18 °C in a freezer for a maximum of 10 d, and then were thawed by applying two thawing conditions (**Figure 1**). The temperature of a cheese sample per freezing/thawing cycle was monitored in the centre and outer part of the cheese using K-type thermocouples (Ni/Al–Ni/Cr) connected to a multimeter (mod. MV100, Yokogawa

Electric Corporation, Tokyo, Japan). As HM Mozzarella cheese is characterized by its high moisture content, rapid drying occurs if the cheese remain separated from its covering liquid; for this reason, after thawing, M_d-treated cheeses were immersed into a freshly prepared covering liquid. All samples were stored at 4 °C for 1 d before analysis.

Trials were performed according to a completely randomized block design. For each batch, fourteen M_d and M_w-cheeses were frozen for each *Fc* (**Figure 1**) in two separate freezing runs. For each *Fc*, the group of frozen cheeses was divided, and 7 cheeses were thawed for each *Tc*. For every batch, measurements were also performed on the fresh, non-frozen cheese that was considered the control, the same day of the treatments' freezing. Before analyses, samples were equilibrated in a climate chamber (mod. ICH 256L, Memmert, Schwabach, Germany) at 25 °C for 1 h.

5.3.2 Physicochemical analyses

Changes in weight caused by the processes were assessed by a laboratory scale (mod. BCE 5200, Orma, Milan, Italy) with an accuracy of ± 0.01 g. Cheeses were weighed before and after freezing and again after the overnight period in a covering liquid after thawing. Changes to the weight were expressed as percentage changes from the original weight.

The moisture Content (**MC**) was measured according to the IDF standard method (1982) by sampling and mixing a whole cheese and performing the analysis in triplicate.

Expressible serum (**ES**) was measured in triplicate by centrifuging 30 g of each sample that was previously cut from a whole cheese in 1-cm cubes, at 12,500 g per 75 min in 50 mL tubes using a benchtop centrifuge (mod. 5810R, Eppendorf, Hamburg, Germany) (**ES_{CT}%**), according to Guo & Kindstedt (1995). After centrifugation, ES_{CT}% was measured after fat layer removal. ES was also measured in quintuplicate by weighing the amount of serum separated after a texture profile analysis (TPA) double compression test (**ES_{TPA}%**). ES_{TPA}% was determined as the weight lost after compressing the sample, similarly to Riebroy, Benjakul, & Visessanguan (2008). The inner part of one whole cheese was cut into five cubes (with 15 mm sides) using a knife. Cubes were placed between double layers of filter papers (type 11106, Sartorius, Goettingen, Germany) and subjected to double compression, as reported in section 2.4. ES_{TPA}% and ES_{CT}% were calculated as the ratio of apparent expressible serum (ES_{app}) weighed using an analytical scale (mod. AR 2140, Ohaus Corporation, New Jersey, USA) to the MC, according to the following equations (1) and (2):

$$\mathrm{ES}_{TPA}\% = \frac{ES_{TPAapp}}{MC} \times 100 \quad (1)$$

$$\mathrm{ES}_{CT}\% = \frac{ES_{CTapp}}{MC} \times 100 \quad (2)$$

The electrical conductivity of ES_{CT}% was measured with a Portamess conductometer (mod. 913, Knick Elektronische, Berlin, Germany) and a TetraCon 325 probe (WTW Xylem Analytics, Weilheim, Germany) having a cell constant (K) of 0.475/cm.

The colour of the inner and outer parts of the cheese were measured using a CR-2600d spectrophotometer (Minolta Co., Osaka, Japan) equipped with a D65 light. The CIE $L^*a^*b^*$ colour space, the lightness of the colour (L^*, from 100 for white to 0 for black), redness (a^*, from +120 for red to -120 for green), yellowness (b^*, from +120 for yellow to -120 for blue) were measured in quintuplicate.

5.3.3 Rheological analysis

Frequency sweep tests were performed using an ARES rheometer (TA instruments, New Castle, USA) equipped with a 25 mm parallel plate geometry according to Alinovi, Cordioli, et al., (2018) with slight modifications. Disk-shaped samples (thickness 4-5 mm, diameter 30 mm) were portioned from the centre of Mozzarella cheese using a slicer and a borer. Sandpaper was applied to the plates to eliminate sample slippage. A solvent trap was used to minimize sample drying during analysis. The temperature during the analysis was set at 25 °C.

Measurements were performed at a constant strain of 0.05% that was into the linear viscoelastic region of the fresh and frozen/thawed cheeses. The frequency dependence of the storage modulus (G'), loss modulus (G'') and complex viscosity (η^*) were evaluated using power-laws equations (3), (4) and (5) (Yilmaz et al., 2016):

$$G' = k'(f)^{n'} \quad (3)$$

$$G'' = k''(f)^{n''} \quad (4)$$

$$\eta^* = k^*(f)^{n^*-1} \quad (5)$$

Measurements were performed in quadruplicate.

5.3.4 Textural analysis

Cheese textural properties were measured at room temperature using a TA.XT2plus texture analyser (Stable Micro Systems, Godalming, UK). Sample preparation was the same as used to measure the ES_{TPA}% (section 2.2). A TPA double compression test was performed using a stainless-steel cylindrical probe with a diameter of 30 mm. A crosshead speed of 1.5 mm/s was applied to compress the cube samples (15 mm side) to 60% strain. The textural parameters considered were hardness (N), cohesiveness, springiness and gumminess (N).

5.3.5 Sensory analysis

Sensory descriptive analysis was performed by eight panellists (5 males, 3 females). A reduced list of cheese descriptors (**Table 1**) from the list from Pagliarini, Monteleone, & Wakeling, (1997) was considered. Panellists were trained by performing 8 training sessions of 1 h to evaluate each descriptor according to three or more reference samples. Scores of the reference samples were adjusted and fixed according to panellists' comments and opinions. The intensity of every Mozzarella sensory descriptor was evaluated between 1 (absence of the attribute) and 9 (extreme intensity of the attribute). After removing the skin, one and a half cheeses were portioned in cubes (with 10 mm sides) for taste and aroma evaluation, while the remaining alf portion was used for visual evaluation. Analyses were performed in duplicate by each panel member by evaluating two of the four batches of cheese.

5.3.6 Statistical analysis

To evaluate the main effects of the freezing/thawing method (M_i; i=1, 2), freezing conditions (Fc_k, k=1, 2, 3) and thawing conditions (Tc_l, l=1, 2), and of their interactions for every measured parameter, split-split plot ANOVA models were created using PRC GLM of SAS (SAS Inst. Inc., NC, USA) according to Alinovi, Rinaldi, & Mucchetti (2018). Batches of cheese (B_j; j=1, 2, 3, 4) were used as the blocking factor of the models (equation 6):

$$Y_{ijkl} = \mu + M_i + B_j + \delta_{ij} + Fc_k + (Fc \times M)_{ik} + \gamma_{ijk} + Tc_l + (M \times Tc)_{il} + (Fc \times Tc)_{kl} + (Fc \times Tc \times M)_{ikl} + \varepsilon_{ijkl} \quad (6)$$

where δ_{ij}, γ_{ijk} and ε_{ijkl} are the main plot and the two subplot error terms, respectively and Y_{ijkl} is the selected response variable. Multiple comparisons (LSD adjustment) were performed among means when significant effects were found and to compare frozen/thawed and control cheeses.

To perform a classification of cheeses based on their characteristics, principal component analysis (PCA) was carried out using normalized variables on two of the four cheese batches. Only important cheese variables were included into the final model, based on their contribution to the definition of PCs calculated from their loadings. Hierarchical cluster analysis (HCA) was carried out on PCs to highlight possible groups of samples based on their PCs scores. Clustering was performed considering Euclidean distances and Ward's method. Pearson's correlation coefficients (r) were also calculated to find relations among evaluated variables. Multivariate analyses were performed using SPSS v.25 (IBM, Armonk, USA).

5.4 Results and discussion

5.4.1 Physicochemical properties

The freezing/thawing methods (*M*) caused significant ($P < 0.05$) weight changes in the cheese (**Table 2)**. M_w-Mozzarella cheeses showed a strong increase in weight (+6.9%), while M_d-Mozzarella cheeses did not show a strong variation of their average weight (100.2%) (**Figure 2A**). Drip losses, moisture evaporation and sublimation during freezing (Delgado & Sun, 2001) caused a weight decrease for M_d-treatments (-2.0%), which was balanced by subsequent rehydration when the cheese was immersed and stored overnight in the covering liquid after thawing. In contrast, M_w-treatments were not affected by weight losses during freezing, thanks to the covering liquid that acts as a glaze (Jaczynski, Tahergorabi, Hunt, & Park, 2016). During thawing, M_w-cheeses absorbed water from the covering liquid and increased their weight.

Accordingly, a significant MC variation ($P < 0.05$) caused by freezing/thawing was associated with these weight changes. M_w-Mozzarella cheeses showed higher MC than the control and M_d-treatments (**Figure 2B**). In contrast, neither *Fc* nor *Tc* had statistical significance in terms of weight variation or MC ($P > 0.05$). The water absorption of M_w-cheeses can be possibly due to the longer freezing (C1: 44.7 ± 3.1 min, C3: 67.3 ± 3.4 min for M_d-cheeses, C1: 116.2 ± 9.1 min, C3: 157.2 ± 7.6 min for M_w-cheeses) and thawing times (S1: 180.1 ± 11.9 min, S2: 309.0 ± 17.8 min for M_d-cheeses, S1: 376.0 ± 84.5 min, S2: 593.0 ± 69.0 min for M_w-cheeses) than for M_d-cheeses. It is well known that lower freezing rates can promote the formation of ice crystals with bigger dimensions that damage the cheese structure. The increased volume of water fraction associated with the formation of ice crystals forces the casein fibres to be in close contact and promotes the formation of bigger serum channels (Bertola et al., 1996; Reid & Yan, 2004). These channels can be responsible for the latter absorption of free water from the covering liquid mediated by capillary forces and by the concentration's gradient of water-soluble molecules (e.g., organic acids, salts, etc.) during the thawing process. During thawing, ice first melts in the covering liquid, generating a zone with a higher temperature and a lower concentration of solutes, promoting the diffusion of water from this zone to the higher solute concentration zone represented by the frozen cheese. In this context, the longer thawing time associated with M_w-cheeses caused by the asymmetry of thermal food properties can improve water absorption (Gonzalez-Sanguinetti, Anon, & Calvelo, 1985). Moreover, changes to the colloidal calcium phosphate content induced by the increasing ionic strength of the medium during

freezing (Kljajevic, et al., 2016) can reduce protein-protein interactions and increase protein hydration (Faccia et al., 2013; Guinee, Feeney, Auty, & Fox, 2002).

The effect of the freezing/thawing processes also led to different results in terms of ES. ES_{CT}% showed higher values than ES_{TPA}%, as the amount of mechanical stress on the samples during centrifugation was strongly higher. ES_{CT}% (**Figure 2C**) and ES_{TPA}% were higher for M_w than for the control and M_d-treatments ($P < 0.05$). Frozen M_w-cheeses showed a lower water holding capacity (WHC), probably because of the minor interactions between caseins and the additional water absorbed from the covering liquid. Related to the damage caused by the formation and growth of ice crystals and the subsequently rearrangement of water into the product, interactions between water and caseins can be modulated by a series of factors, such as the pH and colloidal calcium content, that regulate the hydrophilic and hydrophobic interactions (Faccia, Gambacorta, Natrella, & Caponio, 2019; Faccia et al., 2013) and consequently can have an effect on the tertiary and quaternary casein structure. Freezing can promote casein dehydration phenomena (Reid & Yan, 2004) and an increase of water mobility (Kuo et al., 2003), contributing to the increase of serum separated by the application of physical forces. Additionally, *Fc* was significantly dependant on ES_{CT}% ($P < 0.05$), as the amount of ES separated by the centrifuge method was directly related to the freezing rate, because longer freezing times can promote higher degrees of freezing damage.

Serum collected from M_w-cheeses showed a higher conductivity than from M_d (**Figure 2D**). The lowest conductivity came from M_d serum, which can be explained from M_d-samples being immersed in a freshly prepared covering liquid during the overnight storage after thawing. As the new covering liquid had the same initial composition of the original one, the decrease of conductivity can be caused by salts and organic acids diffusing from the cheese to covering liquid during the refrigerated storage before the freezing-thawing processes (Ghiglietti et al., 2004).

Colour coordinates (L^*, a^*, b^* values) did not exhibit any modification ($P > 0.05$) for all the factors evaluated (**Table 2**). In general, Mozzarella cheese exhibited a high lightness and a dominant yellowish colour, which were respectively higher and lower externally than in the inner part of the cheese ($L^*ext = 93.5 \pm 0.5$, $L^*int = 91.8 \pm 0.5$; $b^*ext = 12.9 \pm 1.56$, $b^*int = 16.0 \pm 1.2$). This was related to the measured higher MC of the outer part (results not shown), as differences in lightness can be caused by different amounts of free water droplets on the analysed surface (Sánchez-Macías et al., 2010).

5.4.2 Rheological properties

Frequency-dependence curves of dynamic moduli (**Figure 3A, B**) highlighted the predominance of the elastic behaviour in all the analysed cheeses, as G' was higher than G'' in the whole frequency range. Both G' and G'' were linearly dependent on the applied frequency variation in a log-log scale. Reported power law equations (equations 3, 4, 5) fitted the experimental rheological data well as the coefficients of determination (R^2) were higher than 0.97.

In the case of G', the power law coefficient k' showed significant differences between cheeses with different freezing/thawing methods (*M*) ($P < 0.05$), despite the differences among the batches (**Table 3**). M_d-treatments were characterized by a higher k' value than M_w-treatments (**Table 4**), while control cheeses were not significantly different from either of the differently treated cheeses ($P > 0.05$). The lower k' of M_w-treatments can be related to their higher MC if compared to the other samples, which can be related to a higher extent of protein hydration caused by calcium phosphate depletion from caseins (Guinee et al., 2002). As water acts as a plasticizer in a viscoelastic system, an increase of its content can promote a decrease in the elastic forces of the cheese body (Alberini et al., 2015; Diefes et al., 1993). Moreover, a decrease to k' can be partially explained by the higher degree of freezing damage caused by the longer freezing and thawing rates. In accordance with the k' variations, differences in the η^* curves were highlighted (**Figure 3D**), as it is also possible to observe from the k^* values (**Table 4**). Moreover, k'' also followed the same trend described for k', despite the estimated P-value for the *M* factor being at the limit of significance ($P = 0.050$). In contrast, no significant main effects relating *Fc* and *Tc* to k', k'' or k^* were highlighted, probably because the freezing and thawing rates considered in this study did not cause a significant modification to the rheological properties.

The frequency dependence of dynamic rheological parameters, which can be estimated from n', n'' and n^* values (**Table 4**), was not influenced by the different process factors, because the slope of the regression curves was not different ($P > 0.05$).

The tangent of the phase angle (tanδ) (**Figure 3C**) confirmed the viscoelasticity of the samples, as it was always lower than 1. Tanδ did not showed differences related to the *M* factor. In contrast, control cheeses exhibited a significantly different tanδ curve than the frozen/thawed cheeses, which can also be observed from the tanδ data reported at a frequency of 1 Hz (**Table 4).** The lower values of tanδ for frozen/thawed cheeses is probably related to the caseins dehydration phenomena already discussed in other studies (Alberini et al., 2015; Alvarenga et al., 2011). During freezing, a

rearrangement of the protein matrix may cause the formation of a more compact texture containing aggregates of casein that interact with each other and are intercalated by serum channels and fat clusters of bigger dimensions. In our case, the increased rigidity seems to not be related to any studied factor or with the different MC of the cheeses but is only related to the application of any freezing or thawing process (**Table 4**).

5.4.3 Textural and sensory properties

Texture analyses partially confirmed the rheological measurements, as M_w-cheeses had a significantly lower hardness and gumminess ($P < 0.05$) than M_d-treated cheeses (**Table 3**). Moreover, as observed with the rheological analyses, control cheeses did not show significant differences between the freezing/thawing treatments (**Table 5**). The cohesiveness and springiness did not show differences related to any factor considered in the models (**Table 3**). However, the control cheese exhibited a significant difference ($P < 0.05$) with the frozen/thawed cheeses for both the cohesiveness and springiness values (**Table 3**). In the case of cohesiveness, the difference was independent of the applied freezing/thawing process, while in the case of springiness the control was different only with M_w-treatments (**Table 5**). Frozen/thawed cheeses showed a lower cohesiveness and a higher springiness than control cheeses. This can be related to changes in the moisture organization subsequent to freezing because of the caseins dehydration phenomena and the formation of a more rigid, less plasticized structure, as the frozen/thawed cheeses had a slightly higher ability to recover their original configuration.

Concerning sensory evaluation, the panel group was not able to detect significant differences related to the freezing/thawing factors for most of the attributes considered ($P > 0.05$) (**Table 3**). Contrary to the textural hardness, sensory hardness was not perceived as being different among the samples., However, as it highlighted a similar trend to textural hardness among the samples, (**Table 5**), the sensory hardness was significantly ($P < 0.01$) but weakly correlated to the TPA hardness, gumminess and MC ($r=0.48$, 0.55 and -0.65, respectively). Accordingly, saltiness was also not perceived as different despite the significantly different electrical conductivity of ES. The only sensory parameter with significant differences related to the *M* factor was juiciness, which showed a significantly lower score for M_d-treatments than control cheese and M_w-treatments. This observation can be related to the ES of the different cheeses, which was the lowest for M_d-cheeses.

5.4.4 Samples classification according to PCA and HCA

Multivariate statistics were considered for an overview and a classification of the cheeses. Sixteen variables were selected from all the measured parameters (**Figure 4B**). PCA generated five PCs that explained 80.47% of the variance of the dataset. The first two PCs used for sample classification and visualization explained the largest amount of variance, at 28.8% and 21.7%. The relatively low variance explained by the model can be caused by batch variability issues as already reported for univariate analyses. However, M_d and M_w-cheeses were clearly classified considering the first two PCs (**Figure 4A**). According to HCA, the control cheese was grouped with M_d-cheeses. In contrast, no classification of the samples based on the different levels of *Fc* and *Tc* was obtained. According to the samples classification, PC1 was mainly represented by negative loadings of textural, rheological and sensory parameters such as TPA and sensory hardness, gumminess, k', and k^* (**Figure 4B**). Parameters such as the weight change, MC, tanδ, ES_{CT}%, ES_{TPA}%, and juiciness showed strong positive loadings on PC1. Interestingly, the sensory and their respective physical parameters showed similar loadings as they were significantly correlated ($P < 0.05$) (saltiness and electrical conductivity, r=0.59; sensory and textural hardness, r=0.48); the only exception was represented by sensory juiciness, which was not correlated with ES but was significantly correlated with weight change (r=0.60) and MC (r=0.44). M_w compared to control and M_d-cheeses was classified according to their lower structural and textural properties and the higher MC and ES.

5.5 Conclusions

Frozen HM Mozzarella cheese is a product increasingly present in both retail and ingredient markets because of its longer shelf-life. Its quality at consumption largely depends on the applied freezing method. HM Mozzarella cheese characteristics were not affected by the freezing and thawing conditions evaluated in this study, however, the presence or absence of a covering liquid during the processes influenced some of the Mozzarella cheese physicochemical, rheological and sensory characteristics. Cheeses processed with a covering liquid were characterized by longer freezing times and showed water absorption phenomena during thawing. Thus, these cheeses were characterized by freezing-induced modifications. In contrast, cheeses frozen/thawed without a covering liquid were more similar to the control cheeses. As a higher moisture content in fresh cheese is often associated with lower storage stability, the results of this study highlighted that freezing and thawing without a covering liquid followed by a rehydration step should be the preferred method to obtain the best results in terms of quality.

5.6 Notes

The article is original and not under consideration by another journal and has not been published previously. The authors declare no conflicts of interest. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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5.9 Tables and figures

Figure 1. Schematic representation of the experimental design to study Mozzarella cheese freezing and thawing processes.

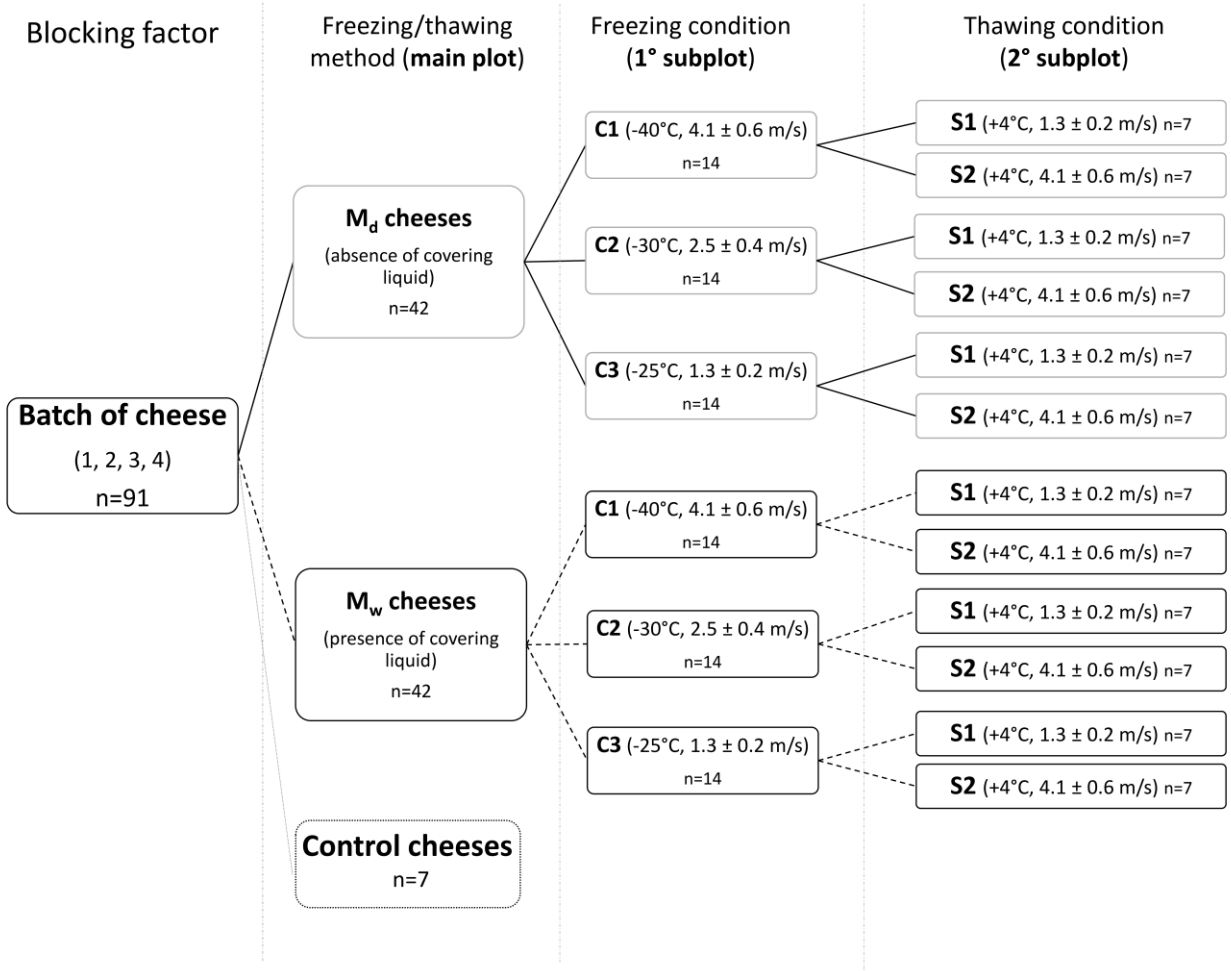

Figure 2. Representation of weight change (A), moisture content (B), expressible serum measured by the centrifuge method (ES_{CT}%) (C), and electrical conductivity (D) of fresh, non-frozen Mozzarella cheese (control) and of frozen Mozzarella cheese without (M_d) and with (M_w) a covering liquid. Columns with different letters are significantly different ($P < 0.05$).

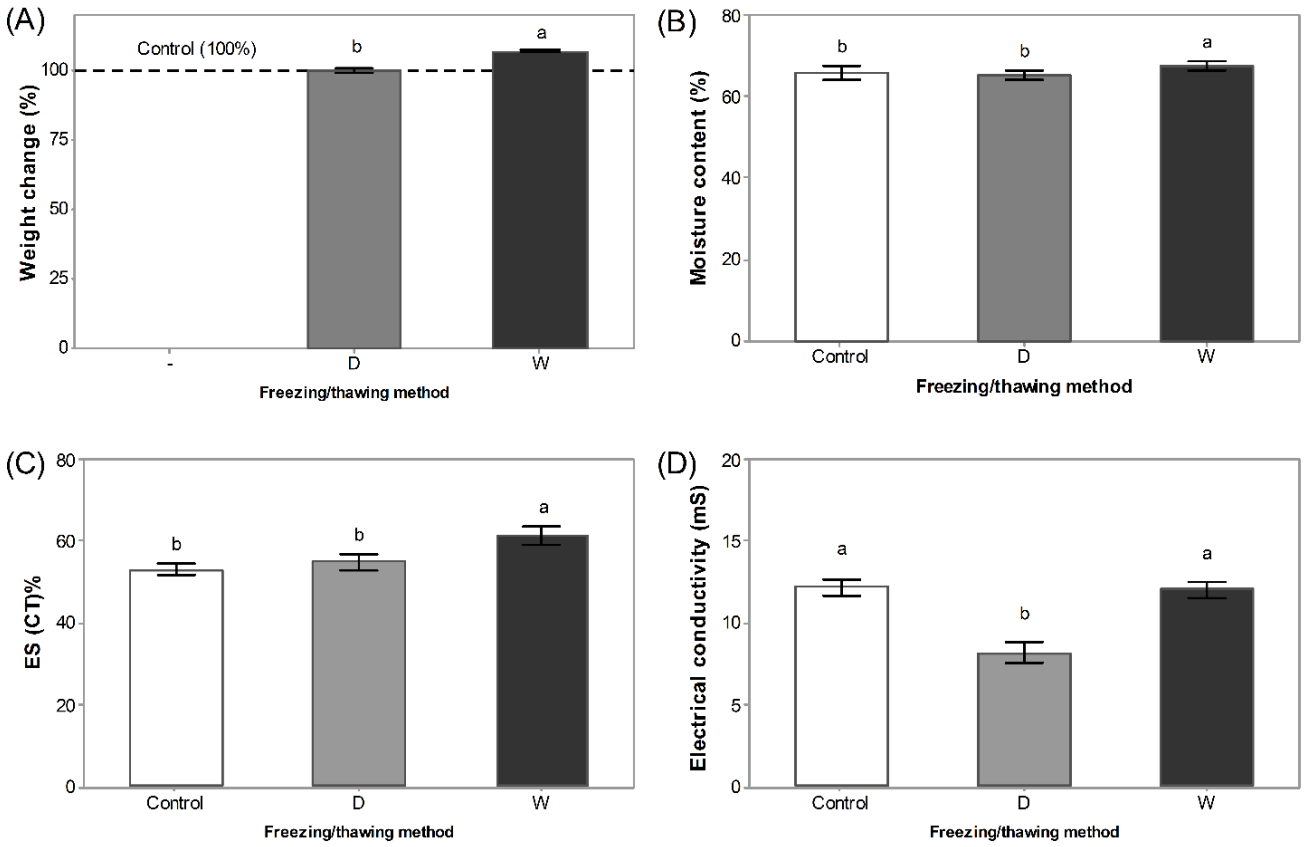

Figure 3. Frequency-dependent dynamic curves of the storage modulus (G') (A), loss modulus (G'') (B), tangent of the phase angle (tanδ) (C), and complex viscosity (η^*) (D) of Mozzarella cheeses. (□) Fresh, non-frozen Mozzarella cheese (control), (◆) frozen Mozzarella cheese without (M_d), and (●) frozen Mozzarella cheese with (M_w) a covering liquid.

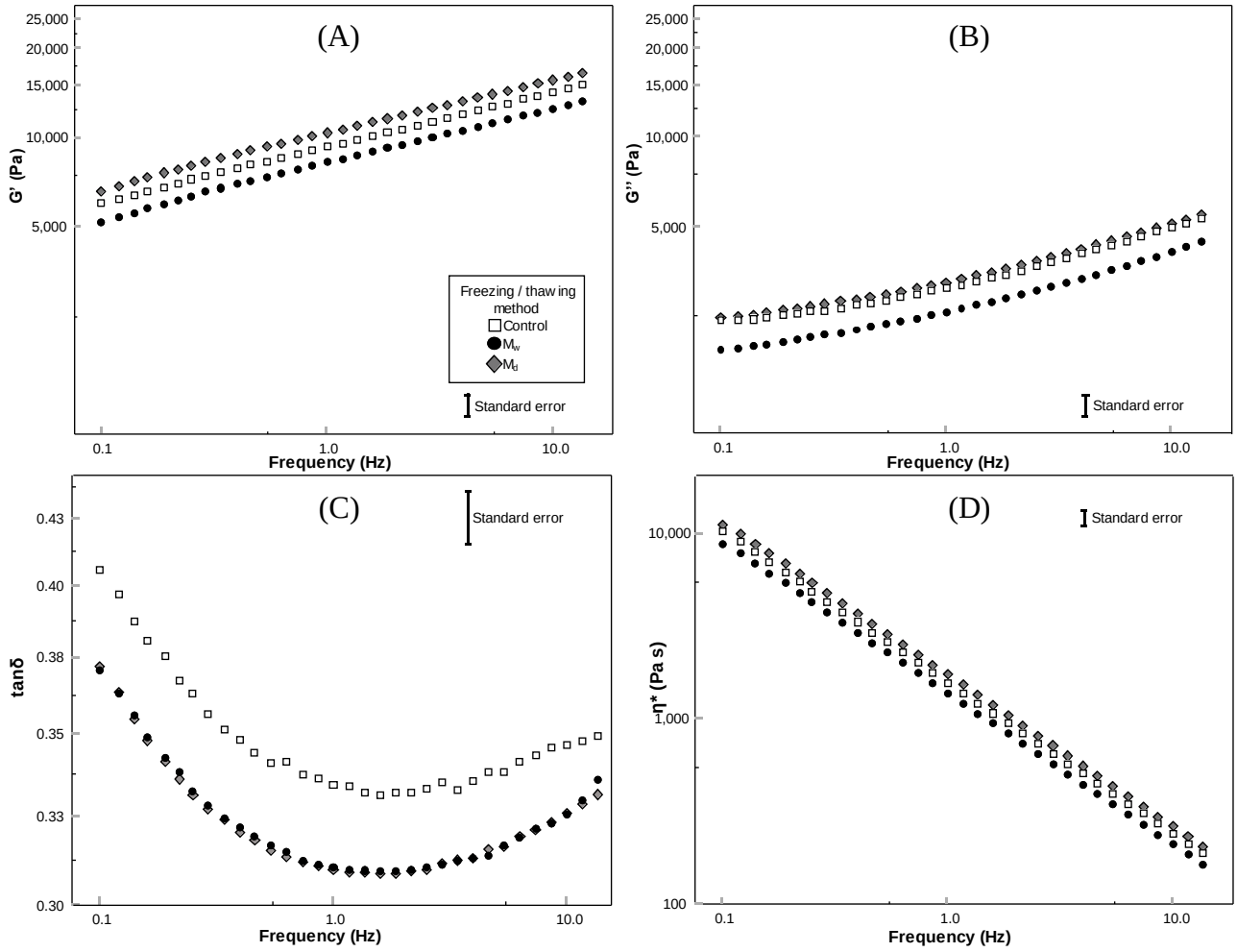

Figure 4. Principal component analysis (PCA) score and loading plots of the first two principal components of the model (PC1, PC2). Principal components were calculated considering a reduced list of chemical, physical, rheological and sensory parameters, and cheese samples were clustered according to the results obtained from the hierarchical cluster analysis based on Euclidean distances and Ward's method. Samples were indicated and labelled according to the different freezing/thawing methods (Control (□): non-frozen cheese; M_d (◆): cheese frozen without covering liquid; M_w (●): cheese frozen with covering liquid), freezing conditions (C1: -40 °C, 4.1 ± 0.6 m/s; C2: -30 °C, 2.5 ± 0.4 m/s; C3: -25 °C, 1.3 ± 0.2 m/s) and thawing conditions (S1: +4 °C, 1.3 ± 0.2 m/s; S2: +4 °C, 4.1 ± 0.6 m/s).

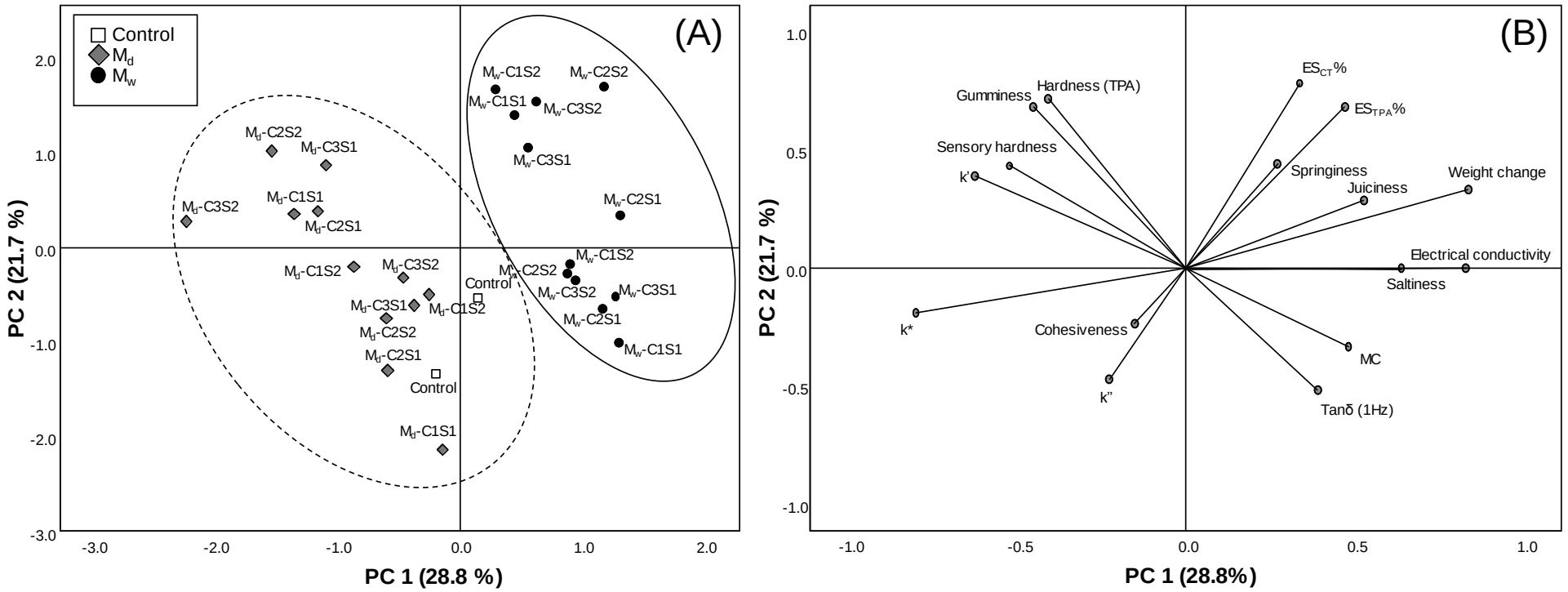

Table 1. Sensory descriptors, meaning and reference samples (with relative scores in brackets) considered during Mozzarella cheese sensory evaluation.

Sensory descriptor	*Meaning*	Reference samples (score)
Texture and taste		
Sensory hardness	*Strength required to compress but not destroy a sample between the molars during the first bite*	Santa Lucia ciliegie-type Mozzarella cheese (1), Conad bocconcini-type Mozzarella cheese (3), Conad 400g-pizza cheese (7), Conad 250g-pizza cheese (9)
Juiciness	*Degree of humidity perceived in the mouth during mastication*	Conad 250g-pizza cheese (1), Conad 400g-pizza cheese (4), Santa Lucia ciliegie-type Mozzarella cheese (6), Vallelata Fiordilatte-type Mozzarella cheese (9)
Acidity	*One of the basic tastes that is perceived on the central part of the tongue*	Conad UHT skimmed milk (0), Conad UHT skimmed milk with 25% of Yomo plain skimmed yogurt (5), onad UHT skimmed milk with 50% of Yomo plain skimmed yogurt (9)
Saltiness	*One of the basic tastes that is perceived on the tip and side parts of the tongue*	Alival citric Mozzarella cheese (1), Alival citric Mozzarella cheese with 0.3% of salt added (5), Alival citric Mozzarella cheese with 0.6% of salt added (9)
Appearance		
Whiteness	*White colour perceived by the human eye*	Conad 400g-pizza cheese (1), Alival citric Mozzarella cheese (3), Alival lactic Mozzarella cheese (9)
Translucency	*Degree of wetness of cheese inner surface*	Conad 400g-pizza cheese (1), Alival citric Mozzarella cheese (5), Alival lactic Mozzarella cheese (9)
Paste smoothness	*Inversely related to the abundance of holes and cracks in the inner part of the cheese*	Alival 5-d old citric Mozzarella cheese (1), Alival 15-d old citric Mozzarella cheese (5), Alival 25-d old citric Mozzarella cheese (7)
Surface smoothness	*Inversely related to the amount of imperfections on the surface of the cheese*	Alival 5-d old citric Mozzarella cheese (1), Alival 15-d old citric Mozzarella cheese (5), Alival 25-d old citric Mozzarella cheese (7)

Table 2. P values obtained from split-split plot ANOVA models of the evaluated physical and chemical parameters for each of the factors considered: Freezing/thawing method (*M*), freezing condition (*Fc*), thawing condition (*Tc*). Measured parameters were the relative weight variation of the cheese (WGT), moisture content (MC), expressible serum measured with centrifuge method (ES_{CT}) and TPA (ES_{TPA}), electrical conductivity of expressible serum (COND) and external and internal colorimetric coordinates (L^*ext, a^*ext, b^*ext and L^*int, a^* int, b^* int).

Parameter	WGT	MC	ES_{CT}	ES_{TPA}	COND	L^*ext	a^*ext	b^*ext	L^*int	a^*int	b^*int
Batch (*Block*)	0.967	0.031	0.057	0.028	0.605	0.202	0.006	0.023	0.034	0.001	0.004
Freezing/Thawing Method (*M*)	0.004	0.042	0.023	0.054	0.009	0.694	0.077	0.930	0.715	0.110	0.306
B x *M*											
Freezing condition (*Fc*)	0.349	0.101	0.044	0.703	0.104	0.501	0.135	0.543	0.615	0.134	0.431
M × *Fc*	0.439	0.606	0.831	0.752	0.003	0.873	0.182	0.586	0.319	0.445	0.613
B x *M* x *Fc*											
Thawing condition (*Tc*)	0.087	0.728	0.328	0.000	0.559	0.597	0.939	0.971	0.084	0.411	0.779
Tc × *M*	0.207	0.544	0.634	0.485	0.988	0.037	0.755	0.080	0.213	0.142	0.814
Tc × *Fc*	0.416	0.128	0.543	0.501	0.074	0.190	0.414	0.326	0.161	0.547	0.082
M × *Fc* × *Tc*	0.103	0.680	0.688	0.888	0.763	0.593	0.219	0.666	0.913	0.080	0.186

Table 3. P values obtained from split-split plot ANOVA models of the evaluated rheological, textural and sensory parameters for each of the factors considered: Freezing/thawing method (*M*), freezing condition (*Fc*), thawing condition (*Tc*). Measured parameters were power law regression parameters from storage modulus (k', n'), loss modulus (k'', n'') and complex viscosity (k^*, n^*); hardness (HAR TPA), cohesiveness (COH), gumminess (GUM), springiness (SPR) measured with TPA double compression; perceived whiteness (WHI), translucency (TRA), hardness (HAR sens), juiciness (JUI), paste and surface smoothness (PAS-SMO, SUR-SMO), acidity (ACI) and saltiness (SAL) from sensory evaluation.

Parameter	k'	k''	k^*	n'	n''	n^*	HAR TPA	COH	GUM	SPR	WHI	TRA	HAR sens	JUI	PAS-SMO	SUR-SMO	ACI	SAL
Batch (*Block*)	0.002	0.061	0.012	0.400	0.912	0.105	0.003	0.138	0.002	0.037	0.443	0.858	0.091	0.108	0.459	0.565	0.564	0.593
Freezing/Thawing Method (*M*)	0.013	0.050	0.024	0.899	0.420	0.349	0.048	0.175	0.028	0.176	0.564	0.352	0.188	0.024	0.176	0.204	0.472	0.236
B x M																		
Freezing condition (*Fc*)	0.338	0.250	0.772	0.967	0.900	0.159	0.989	0.289	0.925	0.131	0.519	0.073	0.284	0.278	0.486	0.241	0.077	0.264
M × *Fc*	0.036	0.796	0.406	0.894	0.125	0.300	0.934	0.265	0.951	0.124	0.556	0.024	0.137	0.968	0.274	0.846	0.215	0.069
B x M x Fc																		
Thawing condition (*Tc*)	0.438	0.075	0.822	0.048	0.733	0.949	0.476	0.797	0.546	0.927	0.537	0.340	0.463	0.017	0.230	0.059	0.143	0.688
Tc × *M*	0.996	0.485	0.271	0.625	0.708	0.469	0.500	0.844	0.506	0.458	0.919	0.411	0.606	0.826	0.852	0.578	0.512	0.691
Tc × *Fc*	0.318	0.491	0.835	0.580	0.905	0.520	0.861	0.064	0.690	0.391	0.431	0.658	0.894	0.299	0.546	0.284	0.941	0.262
M × *Fc* × *Tc*	0.504	0.504	0.137	0.435	0.104	0.906	0.999	0.484	0.954	0.977	0.986	0.529	0.589	0.035	0.947	0.153	0.744	0.748

Table 4. Power law coefficients (mean ± standard deviation) derived from dynamic rheological curves of G', G'' and η^* of control Mozzarella cheese and Mozzarella cheese samples frozen and thawed with (M_w) and without (M_d) covering liquid.

Treatment	k' (Pa s)	k'' (Pa s)	k^* (Pa s)	n' (-)	n'' (-)	n^* (-)	$\tan\delta_{(1Hz)}$ (-)
Control	$9310^{ab} \pm 3952$	$3213^{ab} \pm 1289$	$1566^{ab} \pm 627$	0.1913 ± 0.0204	0.1787 ± 0.0051	0.1932 ± 0.0385	$0.3343^{a} \pm 0.0157$
M_d cheese	$9839^{a} \pm 3819$	$3860^{a} \pm 2076$	$1785^{a} \pm 532$	0.1815 ± 0.0106	0.1750 ± 0.0160	0.1918 ± 0.0143	$0.3106^{b} \pm 0.0193$
M_w cheese	$7897^{b} \pm 3715$	$2836^{b} \pm 1122$	$1344^{b} \pm 631$	0.1822 ± 0.0136	0.1791 ± 0.0116	0.1960 ± 0.0118	$0.3128^{b} \pm 0.0144$

[a-b] Mean values within a column with different superscript letters are significantly different ($P < 0.05$).

Table 5. Textural and sensory parameters of control Mozzarella cheese and Mozzarella cheese samples frozen and thawed with (M_w) and without (M_d) covering liquid. Evaluated parameters were hardness (HAR tpa), cohesiveness (COH), gumminess (GUM), springiness (SPR) measured with TPA double compression; perceived whiteness (WHI), translucency (TRA), hardness (HAR sens), juiciness (JUI), paste and surface smoothness (PAS-SMO, SUR-SMO), acidity (ACI) and saltiness (SAL) from sensory evaluation.

Treat-ment	HAR TPA (N)	COH (-)	GUM (N)	SPR (-)	HAR SENS (-)	JUI (-)	ACI (-)	SAL (-)	WHI (-)	TRA (-)	PAS-SMO (-)	SUR-SMO (-)
Control	$9.12^{ab} \pm 3.93$	$0.65^{a} \pm 0.01$	$5.97^{ab} \pm 2.58$	$0.69^{b} \pm 0.03$	3.6 ± 0.6	$5.1^{a} \pm 1.2$	2.1 ± 0.1	2.8 ± 0.5	2.9 ± 0.5	5.2 ± 0.4	3.1 ± 0.9	4.2 ± 2.0
M_d	$10.07^{a} \pm 2.65$	$0.61^{b} \pm 0.02$	$6.19^{a} \pm 1.69$	$0.71^{ab} \pm 0.05$	4.2 ± 1.1	$3.9^{b} \pm 0.9$	1.8 ± 0.4	2.1 ± 0.4	2.7 ± 0.8	4.9 ± 1.1	3.8 ± 1.4	3.9 ± 0.6
M_w	$9.07^{b} \pm 2.76$	$0.60^{b} \pm 0.02$	$5.51^{b} \pm 1.78$	$0.73^{a} \pm 0.04$	3.5 ± 1.0	$5.4^{a} \pm 1.0$	2.1 ± 0.3	2.8 ± 0.6	2.5 ± 0.9	5.7 ± 1.0	3.1 ± 0.8	4.5 ± 1.2

[a-b] Mean values within a column with different superscript letters are significantly different ($P < 0.05$).

6. Effect of frozen and refrigerated storage on high-moisture Mozzarella cheese quality characteristics

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6.1 Abstract

High-moisture Mozzarella cheese is one of the most exported and consumed Italian cheeses worldwide, but it is affected by low storability. The frozen storage could be effective to improve shelf life and convenience of the product, but its effect has to be estimated. In this study, the effect of prolonged frozen storage and subsequent refrigerated storage after thawing was evaluated over HM Mozzarella cheese characteristics. Frozen cheeses stored at -18°C for a period ranging between 1 and 4 months showed some differences concerning proteolytic phenomena, freezing induced microstructural damages, different water status measured with NMR and its holding capacity, and different textural, sensory properties. Sensory evaluation showed the occurrence of oxidized and bitter tastes, probably because the residual activity of enzymes. The rate of proteolysis of the frozen cheese after thawing was faster than in the fresh cheese and was probably related to dehydration phenomena of caseins induced by freezing temperatures and measured by NMR and the modification in protein structure. These results can be useful to understand the critical point that affects HM Mozzarella cheese quality changes during frozen storage and to find possible ways that can limit the degree of modifications of the matrix.

Keywords: Frozen storage; Mozzarella cheese; Proteolysis; water status; Microstructure

6.2 Introduction

High-moisture Mozzarella cheese is one of the most consumed Italian cheeses worldwide. This kind of product is very different from Mozzarella cheese used as an ingredient for food preparations (pizza, baked products, etc.) as it is usually characterized by a higher moisture content, poor functional characteristics such as shreddability and meltability (Bertola, Califano, Bevilacqua, & Zaritzky, 1996a), and it is usually consumed as a fresh cheese. Moreover, in order to maintain the high moisture of this type of cheese and to avoid the formation of the rind, HM Mozzarella is usually packed into the covering liquid that is a brine containing mono, divalent salts (NaCl, $CaCl_2$) and organic acids.

Italian export of HM Mozzarella cheese has strongly increased over the last years. In 2017 the production of Italian Mozzarella cheese accounted for 313,700 tons, of which 85,136 were exported (Assolatte, 2018), and more than 30% of the total export was imported by extra-European countries; in particular, Mozzarella cheese export is increasing fastly in regions characterized by scarcity of milk (e.g. Asian countries) (CLAL, 2019).

Because of its high moisture content and the fresh taste expected by the consumer, HM Mozzarella cheese is characterized by poor storability; its relatively short shelf life period, ranging from 1 to thirty days (Mucchetti, Pugliese, & Paciulli, 2017), can also have an effect on its commercialization. Fresh Mozzarella cheese products need to be transported with rapid means of transport (e.g. air transport), while slower means of transport (e.g. naval transport) are not applicable to reach long distance markets because of the longer shipping times. However, considering the high costs of transport and the high environmental impact of air transport (Dalla Riva et al., 2017), naval transport would be preferred; in this context, freezing and frozen storage of HM Mozzarella cheese could be a good solution to improve storability of the product, and to create a more sustainable, rational supply chain (Alvarenga, Ferro, Almodôvar, Canada, & Sousa, 2013; Tejada et al., 2000).

HM Mozzarella cheese is not considered a suitable to be frozen in US (USDEC, 2016); despite of that, the freezing process and frozen storage of HM Mozzarella cheese are currently applied in industrial scale and a patent concerning HM Mozzarella cheese freezing has been published (Zambrini & Bernardi, 2017). Conte et al. (2017), compared the effects of freezing rate and 2-months frozen storage of HM Mozzarella cheeses and highlighted a decrease of pores volume and overall sensory quality. Alinovi & Mucchetti (2019) showed the significant effect of different freezing methods (presence or absence of covering liquid during the process) over Mozzarella cheese characteristics but did not highlight a significant effect of freezing and thawing rates, among those tested (from -25

to-40°C with different air velocities). However, there is still a gap of knowledge to be covered; a better understanding of the detrimental phenomena would be useful to highlight and even control the critical points associated to frozen storage of HM Mozzarella cheese and to closely control and tailor the process (Alvarenga et al., 2013).

The application of frozen storage has been largely assessed in the case of low moisture (LM) Mozzarella cheese (Diefes, Rizvi, & Bartsch, 1993; G. G. G. Ribero, Rubiolo, & Zorrilla, 2007; G. G. Ribero, Rubiolo, & Zorrilla, 2009), a pasta filata cheese characterized by lower moisture content and different functional properties that the HM type, and that is mainly used as an ingredient in food preparations.

Frozen storage of cheeses can have an impact over cheese characteristics: it can cause the rupture of the casein matrix, as a consequence of ice crystals formation (Graiver, Zaritzky, & Califano, 2006; Kuo & Gunasekaran, 2009), it can promote protein dehydration and consequently textural and rheological changes (Diefes et al., 1993), and it can modify the sensory perception (Park, Gerard, & Drake, 2006).

Frozen storage has a strong impact in terms of water activity (a_w) of the cheese, as this parameter is strongly affected by temperature and water status. In particular, at temperature of -20°C, a_w can be approximated to 0.82, assuming to be in the range in between the freezing point and the eutectic point of the solution and considering the hypothetical standard state of pure liquid water (Fontana, 2007; Troller & Christian, 1978). In this condition, chemical changes in foods are slowed down and microbial viability is strongly reduced (Troller & Christian, 1978); however, some enzymatic residual activities can still be present at relatively low a_w and temperature (Schmidt, 2007), such as proteolysis and lipolysis and oxidation. Moreover after thawing, the rate of enzymatic reactions in cheese can be even improved as a consequence of ice crystals damage, casein structural modifications, or the liberation of enzymes from microbial cells (Alvarenga, Canada, & Sousa, 2011; Verdini, Zorrilla, & Rubiolo, 2005).

The objective of this work was to evaluate the effects of prolonged frozen storage and subsequent refrigerated storage after thawing over HM Mozzarella cheese characteristics, to assess the applicability of the freezing process to extend the shelf life period of this product and to highlight critical factors that can promote quality changes of the cheese.

6.3 Material and methods

6.3.1 Experimental design

Experimental trials were organized according to a complete block design. Three batches of HM Mozzarella were considered; the batch of cheese, corresponding to cheeses manufactured in different days of cheese making by the same dairy, was assumed as the blocking factor of the design. For each batch, forty-five cheeses were frozen in three separate freezing runs; fifteen cheeses were frozen for each run. To evaluate the effect of the frozen storage on Mozzarella cheese characteristics, a group of fifteen cheeses for each batch was thawed at 1, 3, and 4 months of storage.

Moreover, to also study the effect of refrigerated storage, frozen-thawed cheeses were analyzed during the subsequent refrigerated storage; analyses were carried out at 1, 3 and 8 days after thawing, by subdividing, for each batch, the group of fifteen thawed cheeses in three groups (n=5). For every batch, measurements were also performed on the fresh, non-frozen cheese, that was coded as 0 month of frozen storage, considering the same days of refrigerated storage of the frozen-thawed cheeses (1, 3, 8 days).

6.3.2 Freezing conditions and experiments

Three batches of fresh, HM Mozzarella cheese of 100g were manufactured by Alival S.p.a. (Nuova Castelli S.p.a. RE, Italy) according to the manufacturing method reported by Francolino, Locci, Ghiglietti, Iezzi, & Mucchetti (2010). Manufactured cheeses were kept at 4 ± 1°C into polyethylene bags containing 100g of covering liquid (0.4 % w/w NaCl) for 6 days before being frozen.

Samples were frozen using an air blast freezer (MF 25.1, Irinox, TV, Italy) until a temperature of -20°C was reached in the core of the cheese. Samples were separated from the covering liquid before freezing and process conditions were controlled by imposing an air temperature of -25°C and a velocity of 1.3 ± 0.2 m/s. These conditions were chosen as they did not show a significant impact on cheese quality characteristics (Alinovi & Mucchetti, 2019b) and can be more energy-efficient than faster freezing conditions. Frozen cheeses were immediately vacuum packaged into polyethylene bags and were stored at -18°C.

After reaching the predefined storage times (1, 3, 4 months), samples were thawed into the air blast freezer by applying an air temperature of +4°C, and a velocity of 1.3 ± 0.2 m/s; also in this case, thawing conditions were chosen as they did not show differences in terms of cheese quality characteristics, if compared to faster thawing conditions (Alinovi & Mucchetti, 2019b). After thawing,

cheeses were immersed into freshly prepared covering liquid and were stored in refrigerated conditions (4 ± 1°C). Before being analyzed, samples were taken out of the refrigerator and were equilibrated in a climate chamber (mod. ICH 256L, Memmert, Schwabach, Germany) at 25.0 ± 0.1°C.

6.3.3 Physical and chemical analyses

Changes in weight caused by the processes were measured by a laboratory scale (mod. BCE 5200, Orma, Milan, Italy) with an accuracy of ± 0.1g. Cheeses were weighted before freezing (fresh cheese), immediately after freezing and after thawing, and subsequently the overnight period in 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), while fat and protein content of Mozzarella cheeses were determined using a Tango Near Infrared spectrometer (Bruker, Massachusetts, USA) by mean of commercial calibrations.

Expressible serum of Mozzarella cheese (ES) was measured in triplicate by centrifuging 30g of sample in 50ml falcon tubes at 12,500g per 75min 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 related to the MC of the cheese.

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 costant (K) of 0.475 cm^{-1}.

Colorimetric coordinates of the cheese were measured using a CR-2600d spectrophotometer (Minolta Co., Osaka, Japan) according to CIE $L^*a^*b^*$ color space. Lightness of color (L^*), redness (a^*), yellowness (b^*) were measured in the inner and outer part of the cheese in quintuplicate.

6.3.4 Protein profiling and proteolysis analyses

6.3.4.1 Sample preparation

To perform proteolysis measurements of all samples within a batch in the same laboratory conditions, cheeses were freeze dried using a laboratory equipment (Freeze dryer Lio-5P, 5Pascal, Milano, Italy) and stored at -20°C for a maximum of 4 months.

Freeze-dried samples were finely ground using a mortar; 5g of sample were resuspended in 50 ml of sodium citrate 68mM for fluorescamine assay, while 1g of sample was resuspended in 20 ml of sodium citrate 68mM for electrophoresis analyses and RP-HPLC. Resuspension was performed by mixing the samples using a laboratory homogenizer (ultraturrax T25, IKA, Staufen, Germany) at 14,000 rpm for 4 min. After resuspension, samples were completely rehydrated by keeping it in agitation with a magnetic stirrer at 50°C for 1h (Voutsinas, Katsiari, Pappas, & Mallatou, 1995b).

Finally, samples were skimmed by performing a double centrifugation procedure at 3,000 g for 30 min at 4°C using a benchtop centrifuge (Heraeus multifuge 3 S-R, Hanau, Germany).

6.3.4.1 Reverse-phase HPLC

Reverse phase, high-performance liquid chromatography (RP-HPLC) was performed as already described by Jensen et al., (2012) by separating proteins and polypeptides according to their hydrophobic properties. A volume of 200 µl of cheese solution, having an approximate protein concentration of 2.5g/100ml, was mixed with 600 µl of a solution containing guanidine hydrochloride (6M) and Bis-Tris buffer pH 7 (100 mM), and reduced with 19.5 mM dithioerythritol (DTE). Samples were kept at 37°C for 1 h, centrifuged at 20,800g for 10 min at 7°C and filtered through a 0.45 µm polytetrafluoroethylene filter (Mini-Uniprep, Whatman, Florham Park, NJ).

To perform the analyses, an Agilent LC 1100 series instrument (Agilent Technologies, Santa Clara, CA, USA) was used; the instruments was equipped with a binary pump including degasser, a vial sampler, a column thermostat and a UV diode array detector (G1315A). Aliquots of sample of 6 µl were injected into a Jupiter C4 column (250 mm × 2 mm, 5 µm particle size, 300 Å pore size, Phenomenex, CA, USA), that separated caseins at a controlled temperature of 40°C.

Solvent A was Milli-Q water with 0.05% (vol/vol) trifluoroacetic acid and solvent B was aceto-nitrile with 0.05% (vol/vol) trifluoroacetic acid. The gradient was started at 33% of solvent B and increased up to 50% in 25 min

Caseins were detected and quantified by UV absorbance at 214 nm. Data analysis was conducted using ChemStation software (Agilent Technologies, Santa Clara, CA, USA). Peaks identification was made by comparing retention times with data reported in literature; relative quantification of the caseins and degradation products was made by integrating peak areas and comparing with total integrated peak area within each chromatogram. The relative protein content was calculated as the integrated peak area of a certain compound. All samples were analyzed in duplicate.

6.3.4.2 Polyacrylamide gel electrophoresis analyses

Urea-PAGE was performed according to Andrews (1983) and (Sharma Khanal et al., 2019) using Novex TBE-Urea precast gels (15% total acrylamide, Invitrogen, USA). Sample solutions were mixed with the sample buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.0, 12% Ficoll, 0.01% bromophenol blue, 0.02% xylene cyanole, 7 M urea) in a 1:2 ratio. Sample + sample buffer solutions were heated at 95°C for 5 min and then approximately 5 µg of proteins were loaded into separate wells of the gel. Gels were run at 180 V in a XCell electrophoresis system (Novex, Invitrogen, USA).

Sodium dodecyl sulfate (SDS) PAGE was performed using Mini-PROTEAN TGX precast gels (4–15% acrylamide) that were run on a Mini-Protean II cube (Bio-Rad, Hercules, CA, USA). Samples were diluted in a 1:2 ratio with Laemmli sample buffer (Laemmli, 1970) and were subsequently heated at 95°C for 5 min. Gels were run in non-reducing conditions at 150 V by loading approximately 5 µg of proteins in each well.

All urea and SDS PAGE gels were stained with Coomassie Brilliant Blue G250 according to Blakesley & Boezi, (1977), destained in several changes of distilled water and scanned using ChemiDoc XRS+ (Bio-Rad, Hercules, CA, USA); densitometric analysis was performed using the associated image analysis software (Image Lab v. 5.2.1).

6.3.4.3 Quantification of free amino terminals by fluorescamine assay

Fluorescamine assay was performed according to Dalsgaard, Nielsen, & Larsen (2007) to estimate the formation of peptides and free amino acids by measuring primary amino groups (free N-terminals and lysine side chains) in the samples.

Cheese solutions (5ml) were mixed with an equal volume of 24% trichloroacetic acid (TCA) in a falcon tube and proteins contained in the samples were precipitated at 0°C in ice for 1h. After precipitation, samples, were centrifuged at 13,000 rpm and 4°C for 10min in 2ml eppendorf tubes; 37 µl of supernatant was mixed with 900 µl 0.1M Na-borat ($Na_2B_4O_7$, $10H_2O$) buffer pH 8.0. The resulting solutions were then mixed with 300 µl of 0.2 mg/ml fluorescamine in dried acetone; finally, 250 µl of the obtained solutions were transferred to microwell plates wells of a 96-well white opaque polystyrene plate (Costar 3912, Corning, NY, USA), incubated for 18 min at room temperature, and measured in quadruplicate by fluorescence spectroscopy using a multi-mode microplate reader (Synergy 2, BioTek, VT, USA). Samples were excited at an excitation wavelength of 390 nm and fluorescence emission was acquired at 480 nm using a photomultiplier.

To estimate proteolytic activity, a L-leucine standard curve (0.1-3.0 mM) was built; results were expressed as L-leucine equivalents (mM) in the sample, calculated by linear regression of the standard curve.

6.3.5 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 (10 mm height) were cut from the central part of the cheese and transferred into an NMR tube (outer diameter of 10 mm); 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 times were determined by the inversion-recovery pulse method. The sequence was performed using a the 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.

T_1 and T_2 relaxation curves were fitted with multiexponential models using Sigmaplot, v.10 (Systat Software Inc., USA) according to Gianferri, Maioli, Delfini, & Brosio, (2007) and Diantom et al., (2019), as follows:

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

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

Where $A_1(t)$ $A_2(t)$ 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*, $A_{1(i)}$, $A_{2(i)}$ are the spin-lattice and spin–spin signal intensities, respectively, of component *i*, and the constant L_1 L_2 are the intersects of the polynomial functions 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.

6.3.6 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 a of a 10x objective lens using an Olympus bx51 light microscope (Olympus Corp., Tokyo, Japan). 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).

6.3.7 Rheological analysis

Rheological measurements were performed at a controlled temperature of 25.0 ± 0.1°C using an ARES rheometer (TA instruments, New Castle, USA); the instrument was equipped with a 25 mm parallel plate geometry with sandpaper to avoid sample slippage, and with a solvent trap to avoid moisture losses.

Analyses were performed in quadruplicate according to (Alinovi, Cordioli, et al., 2018) with slight modifications. Disk-shape samples (thickness 4 mm, diameter 30 mm) were portioned from the central part of Mozzarella cheese using a slicer and a borer. Strain sweep tests were performed in order to measure the linear viscoelastic region of the samples. A strain value of 0.05% was found to be comprised into the linear viscoelastic range of all the samples and was used to perform frequency sweep tests. Frequency-dependence of storage modulus (G'), loss modulus (G'') and complex viscosity (η^*) were evaluated using power-laws equations (Steffe, 1996):

$$G' = k'(f)^{n''} \quad (3)$$

$$G'' = k''(f)^{n''} \quad (4)$$

$$\eta^* = k^*(f)^{n^*-1} \quad (5)$$

6.3.8 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).

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).

6.3.9 Descriptive sensory analysis

Quantitative descriptive analysis was performed by five panelists (three males, two females) that were trained according to Alinovi & Mucchetti (2019), and that had previous experience with descriptive sensory analysis of Mozzarella cheese. Evaluated sensory descriptors were chosen according to a reduced list of cheese descriptors from the list of Pagliarini, Monteleone, & Wakeling (1997). Eight descriptors were considered as reported and described in a previous work (Alinovi & Mucchetti, 2019b): sensory hardness, juiciness, acidity, saltiness, whiteness, translucency, paste smoothness, surface smoothness. Additionally, two new descriptors, bitterness and intensity of oxidized, were also included to possibly observe the appearance of off-flavors caused by the residual activity of enzymes or chemical reactions. The intensity of every descriptor was evaluated between 1 (absence of the attribute) and 9 (extreme intensity of the attribute). Cheeses were portioned in 10 mm cubes for taste and aroma evaluation, while a half portion of the cheeses was used for visual evaluation.

6.3.10 Statistical analysis

To evaluate 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, split-plot ANOVA models were created for all the parameters evaluated using PRC GLM of SAS (SAS Inst. Inc., NC, USA) according to Alinovi, Rinaldi, & Mucchetti (2018). Batch of cheese (B_j, j=1, 2, 3) was used as the blocking factor of the models (equation 4):

$$Y_{ijkl} = \mu + Ft_i + B_j + \delta_{ij} + Rt_k + (Ft \times Rt)_{ik} + \gamma_{ijk} \quad (6)$$

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.

To investigate about sample classification using a multivariate approach, principal component analysis (PCA) was also performed. Prior to analysis, variables were normalized; parameters were screened on the basis of their contribution in the definition of PCs calculated from their loadings, and only important variables were included into the final model.

Hierarchical cluster analysis (HCA) was carried out on PCs to highlight possible groups of samples based on their PCs scores; clustering was performed considering squared Euclidean distances and

Ward's method. Pearson's correlation coefficients (r) were also calculated to find relations among evaluated variables. Multivariate analyses were performed using SPSS v.25 (IBM, Armonk, USA).

6.4 Results and discussion

6.4.1 Physical and chemical characteristics

Frozen Mozzarella cheeses showed a weight decrease (residual weight of 98.4 ± 0.2%, compared to the original fresh cheese weight) subsequently freezing, that was further enhanced by the thawing process after frozen storage (97.5 ± 0.7%). The variation of weight was however not influenced by the frozen storage time (P=0.690). After the overnight period, frozen-thawed cheeses regained weight as a consequence of water absorption from the covering liquid; as a consequence, weight difference between fresh cheeses weighted before freezing and frozen-stored cheeses subjected to the overnight period in covering liquid after thawing, was negligible (residual weight of 100.0 ± 1.0%), according with the results of a previous work (Alinovi & Mucchetti, 2019b). Weight change after the overnight period was also not influenced by the frozen storage period (P=0.718).

Accordingly, HM Mozzarella cheese chemical composition was not influenced by *Ft* (P>0.05), as moisture, fat, and protein content did not change over frozen storage (Table 1); this observation showed the ability of the skin, vacuum packaging to avoid ice sublimation during storage. Also, considering *Rt* it was not possible to highlight a significant variation of the chemical composition (P>0.05). Considering the gross composition, the only factor in the statistical models showing significant variability was the blocking factor (batch) (Table 1); despite the cheesemaking process was standardized, the degree of standardization mainly led to obtain the control of pH and ES parameters, while all the other measured parameters showed differences among batches.

The fraction of unbound water contained in Mozzarella cheese, estimated by ES, showed a significant increase during the frozen storage period; already from the first month of frozen storage, the amount of ES was significantly higher (+3.5%) than the control fresh cheese (Table 2). An increase of ES consequently to frozen storage is a clear index of water rearrangement phenomena caused by the storage period; as frozen storage time increases, casein dehydration phenomena, that can be a consequence of the formation, reaggregation of ice crystals (Kuo & Gunasekaran, 2009) and of conformational changes in protein structure (Fontecha, Bellanato, & Juarez, 1993), can have a stronger impact over water status also from a macroscopic point of view. During frozen storage, water released from the casein matrix migrates to form larger serum channels; the result of this

phenomenon is the decrease of bound water and the consequent increase of unbound water. In facts, after thawing, the dense protein network is no longer able to re-adsorb bulk water, diminishing its lubricant effect and thus producing a harder cheese structure (Bertola, Califano, Bevilacqua, & Zaritzky, 1996b).

The mean electrical conductivity of ES did not show a particular trend and a significant effect for both *Ft* and *Rt* variables (Table 2). On the contrary, the decrease of pH of ES during frozen storage was at the limit of significance (P=0.05) (Table 1). This variation of pH can be justified by the possible precipitation of insoluble calcium phosphate as a consequence of frozen storage that can increase the ionic strength, and consequently decrease the buffering capacity and pH of the medium; this was also found to be related to caseins precipitation phenomena caused by changes in ionic equilibria (Kljajevic et al., 2016; Van Den Berg, 1961). However, this phenomenon was not clearly observable in our case, as the electrical conductivity, that can be related both to the ionic strength and the organic acids content (Mucchetti, Gatti, & Neviani, 1994), did not change significantly, and pH decrease was small.

Lightness (L^*) showed a significant decrease at longer frozen storage times, both in the inner and in the outer part of the cheese ($P < 0.05$). A decrease of L^* can be caused by a differences in the amount and distribution of free water droplets on the analyzed surface (Sánchez-Macías et al., 2010) but can also be related to an increase of the degree of oxidation of the cheese lipid phase and non-enzymatic browning resulting from oxidation products and amino acids (Mahajan, Bhat, & Kumar, 2015; Trobetas, Badeka, & Kontominas, 2008). Moreover, the higher extent of oxidation of the cheese at longer *Ft* can be confirmed by the increase in b^* values, that was significant in the outer part (Table 1). On the contrary, a^* coordinate showed a significant effect and an increase after 8 days of refrigerated storage, but it did not show a significant effect of *Ft*. In general, Mozzarella cheese exhibited a high lightness and a dominant yellowish color, that were respectively higher and lower in the external than in the inner part of the cheese, according to the results of Alinovi & Mucchetti (2019).

6.4.2 Characterization of protein profile by SDS and urea PAGE

Results of the SDS-PAGE analysis of HM Mozzarella cheese at different *Ft* and *Rt* are reported in the case of batch 1, in figure 1. As it can be observed, the electrophoresis analysis of HM Mozzarella cheese samples did not show a good separation among the main caseins (CNs), β-CN and α_s-CNs (bands located in the range between 29–34 kDa), with the exception of para-κ-CN, that is characterized by a very different molecular weight (MW), around 13 kDa. On the contrary, it was possible to clearly distinguish the higher MW fractions and the lower MW fractions, corresponding to the CN degradation products.

All Mozzarella cheese samples were characterized by high MW fractions that can be attributed to α_{s2}-CN dimers (Nielsen, Purup, & Larsen, 2019), whey protein aggregates (Galani & Apenten, 1999), or whey protein-CN complexes linked by disulfide bonds (Havea, Carr, & Creamer, 2004) that can form during cheese making as a consequence of temperature reached during the pasteurization and stretching step.

Several low MW degradation products can be observed from the electrophoretogram that can be ascribed to an early proteolytic activity in milk before cheese making and in cheese during the 7-days storage period at 4°C before experiments; on the contrary, during both *Rt* and *Ft* periods, it was not possible to highlight an evident proteolysis, as the bands corresponding to CNs and high MW aggregates did not show a decrease of intensity; and the low MW did not show a strong increase. Among the others low MW fractions, it was possible to identify γ_1, γ_2, γ_3, and β-CN (f69–209) (γ_4), according to literature data (Di Luccia et al., 2009; Petrella et al., 2015; Somma, Ferranti, Addeo, Mauriello, & Chianese, 2008). Only a slight increase of intensity can be observed at longer frozen storage times for γ_1, γ_2, γ_3-CNs.

Urea-PAGE analysis results can be observed in figure 2, in the case of batch 1 cheeses for all *Ft* and *Rt* considered conditions. This analysis showed the presence of the major CNs (β-CN, α_{s1}-CNs and α_{s2}-CNs), that were better separated than with SDS-PAGE. According to literature data, it was possible to detect bands corresponding to γ_1, γ_2, γ_3-CNs, α_{s1}-I (f24-199) and α_{s1} low MW degradation products (Costabel, Pauletti, & Hynes, 2007; Sharma Khanal et al., 2019).

As it is possible to observe, there was a slight increase of γ_1, γ_2, γ_3-CNs concentration at long frozen and refrigerated storage times, that can be related to the activity of residual plasmin in the cheese (Costabel et al., 2007); as the concentration of γ-CNs did not show an increase in the case of the fresh, non-frozen cheese during refrigerated storage (lanes 0.1, 0.3, 0.8), it can be hypothesized that

the frozen stored casein matrix becomes more susceptible to be proteolyzed during the subsequent refrigerated storage period (Bertola et al., 1996b); accordingly, also α_{s1} hydrolysis followed this trend, and lower MW products were found from the first month of *Ft*. However, the extent of primary proteolysis was not high, as the decrease of intensity of bands related to CNs was not minimal.

6.4.3 Evaluation of proteolysis by reverse phase HPLC and fluorescamine assay

Chromatographic analyses separated the major caseins fractions para-κ-CN, α_{s2}-CN, α_{s1}-CN and β-CN, with α_{s1}-CN and β-CN that were also separated on the basis of their different genetic variant: α_{s1}-CN-8P and α_{s1}-CN-9P, and β-CN A1, β-CN A2 and β-CN B (Bijl et al., 2014; Frederiksen et al., 2011).

Minor peaks, marked in figure 3 as peak 1, 2 and 3, were also separated and corresponded to degradation products of CNs (Jansson et al., 2014; Nielsen et al., 2018; Zhang, Bijl, & Hettinga, 2018) with peak 1 that can be mainly related to α_{s1}-CN degradation products and peak 3 to γ-CNs (Rauh, 2014); these peaks were already present in control cheese, as a consequence of early proteolysis phenomena. In accordance with urea-PAGE electrophoresis results, it was possible to observe a slight increase of degradation products peak's area at longer *Ft* and *Rt* storage times (figure 4).

From the obtained results of the ANOVA models (Table 3), it was possible to highlight a significant effect of *Ft* and *Rt* over the relative percentage of degradation products 1 and 3, and the effect of *Rt* over degradation product 2 and β-CN percentage decrease (Table 4). On the contrary, the was no significant difference over α_{s1}-CN, α_{s2}-CN and para-κ-CN relative percentage. Accordingly, it can be hypothesized that the main substrate for proteolytic enzymes was β-CN, that can be mainly hydrolyzed into γ-CNs by the activity of plasmin, (Kelly & McSweeney, 2003), that is not completely inactivated by milk pasteurization and curd stretching operations. Heat stresses, such those occurring during HM Mozzarella cheese-making process, can promote the conversion of plasminogen to plasmin, because of the inactivation of the inhibitors of this reaction (Lucey, Johnson, & Horne, 2003). Among the three CN degradation products, degradation product 3, showed significant interaction between *Ft* and *Rt*, that is the consequence of the different proteolysis rate caused by different frozen and refrigerated storage times. In particular, longer times of frozen storage determined a higher rate of proteolysis during the subsequent refrigerated storage (Figure 5), also in accordance with observations made with urea PAGE. This phenomenon, as previously discussed, can be addressed to casein dehydration phenomena and to freeze-induced modification of tertiary and quaternary casein structure during prolonged frozen storage (Alvarenga et al., 2013) that can promote the activity of

plasmin and other indigenous enzymes and the accessibility of the enzymes to the substrate (Verdini et al., 2005).

This result was also in accordance with the estimation of free amino groups made by the fluorescamine assay (Figure 6), that give an overview of the extent of proteolysis in the sample, and showed a similar trend and the significant effect of *Ft*, *Rt* and *Ft* × *Rt*.

However, despite of the significant increase of proteolysis during *Ft* and *Rt* periods, measured with HPLC and fluorescamine assay, and also observed with urea PAGE, the extent of CN degradation was relatively low; for example, the decrease of β-CN measured with reverse phase HPLC was around 4.5%, by comparing the control cheese at 1 day of *Rt* and the 4-months frozen stored cheese at 1 day of *Rt*, and 10%, by comparing the control cheese at 1 day of *Rt* and the 4-months frozen stored cheese at 8 days of *Rt*.

6.4.4 ^{1}H T_1, T_2 NMR results

A visual representation of ^{1}H T_1 relaxation curves is reported in figure 7. As it is possible to observe, longitudinal relaxation curves, were characterized by the presence of two ^{1}H populations that showed completely different relaxation times and relative percentages. The fastest relaxing component, population A, was characterized by a relaxation time ranging between 0.9 and 2.6 ms, and a relative percentage ranging between 22.4 and 3.4 %, while the slowest relaxing component, population B, was characterized by a relaxation time ranging between 176 and 381 ms. and a relative percentage ranging between 22.4 and 3.6 %, 77.6 and 94.4%. Thus, relaxation components A and B were respectively, tentatively assigned to protons of water molecules that are bounded or unbounded to macromolecules such as caseins but can also be affected by the presence and the state of the fat phase. Therefore, microstructural, chemical changes related to the frozen and the refrigerated storage (i.e. freeze-induced modification of the casein matrix, fat globules clustering and agglomeration, serum channels modifications) can have also an impact over ^{1}H relaxation and chemical exchange processes (Gianferri, Maioli, et al., 2007).

Accordingly, longitudinal relaxation curves showed also some clear differences as a function of both *Ft* and *Rt* (Table 5). At longer *Ft*, relative abundance of population A significantly decreased (Figure 9), while relative percentage of population B consequently increased. This phenomenon can be related to the migration of water molecules that are strongly bonded to the casein structure, to chemical groups containing reactive protons, or trapped into the casein matrix (Gianferri, Maioli, et

al., 2007), to the interstitial voids that are surrounded by the protein domain (serum channels). This migration of water can be related to protein dehydration phenomena, as already reported before; moreover, the percentage increase of population B was also significantly related to the increase of ES during the frozen storage period (r=0.380).

Considering T_1 relaxation times, it was possible to observe the significant effect of *Rt* on the molecular rigidity of population B. In facts, the slowest relaxing component shifted to longer relaxation times during the refrigerated storage, as the mean ^1H T_{1B} at the first day of refrigerated storage was 231 ± 50 ms, while after 8 days of refrigerated storage became 288 ± 48 ms. A similar increase of ^1H T_1 was also reported by Kuo, Gunasekaran, Johnson, & Chen, (2001); they hypothesized that an increase in relaxation time in the early storage period can be related to proteins structural rearrangements, because of the dynamic, non-quiescent state of caseins after stretching and molding.

In this study, the phenomenon of protein hydration that can be normally observed during the refrigerated storage period of LM Mozzarella cheeses (Guo & Kindstedt, 1995; Kuo & Gunasekaran, 2009; Kuo et al., 2001), was not highlighted; the different behavior observed in our study, confirmed by both NMR and ES measurements, can be related to the extent of freezing induced damage to the caseins, that can promote modification of the tertiary and quaternary structure of proteins (Xiong, 1997) and irreversible dehydration; moreover, also proteolytic phenomena, that were previously described can have an impact.

Differently from LM Mozzarella that is not stored into a brine, HM Mozzarella was kept into covering liquid (0.4% NaCl w/w) for the *Rt* period, determining a recovery of the weight lost during freezing. The behavior of the absorbed water (about 3.5% of total moisture of the cheese) may be completely different from that of the original water of Mozzarella cheese, as it did not appear to interact with casein and fat, being easily separated as ES.

^1H T_2 relaxation curves showed the presence of four ^1H populations, according to (Gianferri, D'Aiuto, Curini, Delfini, & Brosio, 2007); the spin-spin relaxometry can give different information than spin-echo relaxometry, because it involves additional relaxation mechanisms (i.e., exchange of nuclei between different environments) (Kuo et al., 2001; Lucas, Wagener, Barey, & Mariette, 2005), and it can give a different separation of ^1H components.

The three longer relaxation components (populations B, C, D) were partially overlapped, while the only completely resolved population (A), was the most rigid one, that corresponds to the ^1H of water strongly bound to the casein structure as solvation water (Figure 8). During *Ft*, the overlapping zones

of populations B, C, D increased, indicating a system that from a molecular point of view is becoming more homogeneous.

Population A showed a significant increase of its relaxation time with both *Ft* and *Rt* (Figure 10a, b), while its intensity did not change (Table 5); this increase could be related to the extent of proteolytic phenomena and the depletion of peptides, as T_{2A} was found to be related to the increase of CNs degradation products (e.g. r=0.501 for peak 3, and r=0.512 for peak 1).

Components B and C, that were assigned by Gianferri, D'Aiuto, et al., (2007) to the water trapped in the protein meshes of Mozzarella di Bufala Campana cheese and to the lipids contained into the fat globules, respectively. However, characteristics of buffalo Mozzarella cheese are quite different from HM Mozzarella for the higher fat/caseins ratio (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 8); thus, we tentatively hypothesized that both components were constituted by ^1H related to the fat phase and to the water molecules. At longer *Ft* and *Rt*, component B decreased its relative percentage, while component C increased it (Figure 10c, d) presumably because of water/fat phase being less bound and entrapped by the casein matrix, 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 of *Rt*, to 77 ± 5 ms at day 8th day of *Rt*.

6.4.5 Microstructural changes

Microstructural changes of HM Mozzarella cheeses during frozen storage at different periods can be observed in figure 9. Microstructure of the cheese already changed from the 1 month of frozen storage at -18°C, if 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 globules clusters and larger cavities surrounded by the protein matrix. These results are in accordance to the observed increase of ES (Table 2), to the modifications of the molecular status of water observed by ^1H T_1 and ^1H T_1 NMR relaxometry, and to microstructural observations made by Kuo & Gunasekaran (2009) and (Graiver et al., 2006), that observed an increase in pores size and breaks of the protein structure 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 cavities 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).

6.4.6 Rheological and textural properties

Frequency curves of dynamic moduli (G', G'') showed the predominance of the elastic behavior in Mozzarella cheeses, as G' was higher than G'' in the whole frequency range. Both G' and G'' were linearly dependent to the applied frequency variation in a log-log scale, and were well-fitted by power law equations (equations 3, 4) ($R^2 > 0.96$).

As it is reported in table 6, none of the estimated regression coefficients (k', k'', k^*, n', n'', n^*) showed significant effects of main factors Ft and Rt, and of their interaction (P>0.05). The only factor in the statistical models that showed significance only for k' and k" coefficients was the blocking factor (batch); despite the cheesemaking procedure was standardized as previously discussed, differences also in terms of rheological behavior of obtained cheeses were still appreciable. Considering these results, the freezing process and the frozen storage and refrigerated storage applied did not change the rheological behavior of the cheeses.

On the contrary, textural analyses showed a significant variation of hardness and gumminess as a function of *Ft* (P<0.05, table 6). 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 7). The increase of hardness and gumminess until the third month of frozen storage can be related to the already discussed protein dehydration phenomena; frozen storage can promote rearrangement and the 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 et al., 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 the higher extent of protein hydrolysis, as already discussed earlier and by other authors (Meza, Verdini, & Rubiolo, 2011).

Also, cohesiveness showed the significant effect of frozen storage: fresh, non-frozen cheeses were found to be different from the frozen cheeses, already from the first month of frozen storage. This result was in accordance with findings from a previous work (Alinovi & Mucchetti, 2019b); in this case, the higher cohesiveness of control cheese than frozen-stored cheeses can be related to changes in microstructure and moisture organization subsequently freezing and frozen storage, because of caseins dehydration phenomena, freeze-induced reorganization and damage of the casein matrix, and the consequent formation of a more plasticized structure. Accordingly, cohesiveness was found to be negatively correlated to the proteolytic phenomena (e.g. with the formation of peak 1, r=-0.516), and to protein dehydration phenomena (e.g. with NMR T_1 population B, r=-0.415)

6.4.7 Sensory properties

From a sensory point of view, cheeses showed difference concerning the appearance of surface smoothness, and the intensity of bitter and oxidized tastes, while the other parameters were not affected by both *Ft* and *Rt* (Table 8).

In particular, it was possible to observe a significant increase of the amount of imperfections on the surface of the cheese during the 4-month frozen storage period (Figure 11) already from the first month of frozen storage (Figure 12a). This modification can be caused by peel off phenomena of the cheese's surface, that is usually reported after prolonged refrigerated storage of HM Mozzarella cheese into covering liquid (Laurienzo et al., 2008) and that can be enhanced by the caseinolytic activity of some non-lactic acid bacteria directly on the cheese surface (Baruzzi, Lagonigro, Quintieri, Morea, & Caputo, 2012). In this case, skin separation phenomena can be related to the abrupt change of thermal and physical properties as a consequence of temperature changes and fluctuations during freezing, thawing and storage, and to temperature non-homogeneities in the outer part of the cheese, that are a consequence of Mozzarella cheese non-regular geometry (Alinovi & Mucchetti, 2019a). In particular, it can be hypothesized that 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 is lost in the covering liquid.

As said, frozen storage promoted also the formation of oxidized and bitter tastes, with the first one that was found to be significant already from the first month of frozen storage, while the second sensory attribute was significantly higher from the third month (Figure 12c, d).

The increase in bitterness of the cheese can be related to the increase in proteolysis during the frozen storage period ($r > 0.500$ with degradation products measured with HPLC); it is well known that the depletion of peptides (in particular hydrophobic fragments) from caseins can promote the formation of bitter tastes (Alinovi, Cordioli, et al., 2018). In particular, the residual activity of indigenous proteases, such as plasmin, or microbial proteases can liberate potentially bitter peptides from αs_1-CN (e.g. f23–34, f91–100, f100–105), that were found also in HM Mozzarella cheese (Faccia et al., 2014), and from β-CN (e.g. f193-209, f106-113, f190-209) or αs_2-CN (e.g. f171-181, f182-207, f189-207) (Fox & McSweeney, 1997; Rauh et al., 2014; Sousa, Ardö, & McSweeney, 2001).

In the same way, the appearance of oxidized tastes in HM Mozzarella cheese can be mainly caused by the residual activity of endogenous and microbial lipases and the presence of oxygen, that can penetrate through the packaging material. It has been reported that frozen storage induces a significant deactivation of lipase enzymes in sheep's milk, but without completely deactivating it (Needs, 1992). Also ice crystal growth during frozen storage can contribute to the higher extent of oxidative phenomena, as it can cause the rupture of fat globule membranes, release of acylglycerols in the matrix that can be subsequently hydrolyzed in fatty acids and become more propense to oxidation (Voutsinas, Katsiari, Pappas, & Mallatou, 1995a). The presence of oxidative phenomena in this study was also indirectly confirmed by changes in the color of the cheese, that was significantly correlated ($r=-0.457$ and $r=0.444$ with L^* ext and b^* ext, respectively), as previously reported. On the contrary, as a consequence of the frozen storage period without illumination, the extent of photo-induced oxidation would be low.

Moreover, concerning the refrigerated storage period considered, it was possible to highlight a decrease of sensory hardness, that was significant after 7 days of storage (Figure 12b), and that can be related to casein breakdown caused by proteolytic enzymes; in particular, the depletion of αs_1-CN f24-199 is recognized to be one of the main contributors to cheese softening (Alinovi, Cordioli, et al., 2018), and showed an increase of its concentration during the refrigerated storage, as previously reported in urea-PAGE results. However, the decrease of sensory hardness was not statistically confirmed by texture analysis measurements, that did not show a significant effect of *Rt* (Table 6); however, also textural hardness showed a slight, non-significant decrease (-1.0 N from 1 day to 8 days of *Rt*), and the calculated correlation between sensory and textural hardness was good ($r=0.750$).

6.4.8 Samples classification according to PCA and HCA

Multivariate statistics (PCA and HCA) were performed to have an overview and a classification of the cheeses. Of the totality of measured parameters, thirty variables were selected to perform PCA (Figure 13a), and to generate three PCs that explained 64.1% of variance of the dataset. The low variance explained by the multivariate model can due to variability encountered in relation with the batch of cheese, as already observed in the case of univariate analyses, and because the process evaluated variables (*Ft* and *Rt*) did not show a strong influence over many measured parameters.

However, considering a multivariate approach, a good discrimination between fresh and frozen stored-cheeses can be observed (Figure 13b); according to HCA, all the fresh cheeses were grouped together, while frozen stored cheeses were not further classified as a function of the frozen storage period. Moreover, as it is possible to observe in figure 13c, *Rt* did not cluster the cheeses in relation of the measured parameters.

By observing the loading plot (Figure 13a), PC1 was mainly represented by positive loadings of proteolysis degradation products and NMR T_2 relaxation times and percentages of mobile water fractions. On the contrary, percentage of β-CN, textural and rheological parameters, and the relative percentage of T_2 more rigid proton populations showed negative loading of PC1. PC2 was determined by positive loadings of b^* ext, oxidized and bitter tastes, expressible serum, and T_1 unbound proton population (B), and by negative loading of pH of ES, lightness (L^*), juiciness and the percentage of the less rigid T_2 population.

It is interesting to observe a good correlation between sensory juiciness and the relative percentage of the more mobile T_2 D population (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.

Moreover, despite it did not show significant differences, moisture content was inversely correlated, as expected, with textural, sensory hardness and k' (r=-0.640, -0.617, -0.740, respectively).

By comparing the loading and score plots, fresh cheeses were mainly differentiated from the frozen thawed cheeses for their lower proteolysis, different water status and mobility, less oxidized and bitter sensory perception, different color and higher cohesiveness.

6.5 Conclusions

Frozen HM Mozzarella cheeses stored at -18°C for a period ranging between 1 and 4 months showed higher proteolysis phenomena, freezing induced microstructural damages, different water status and holding capacity, and different textural, sensory properties than fresh Mozzarella cheeses, evaluated within 7 days – 15 days after cheesemaking.

The residual activity of enzymes during frozen storage can be responsible for the occurrence of oxidized and bitter tastes. Moreover, the enhanced rate of proteolysis after thawing, primarily caused by plasmin, was probably found to be related to dehydration phenomena of caseins induced by freezing temperatures. These are critical points that must be considered when storing HM Mozzarella cheese in freezing conditions, as they can considerably reduce the refrigerated shelf life after thawing, and product's sensory quality.

These results can be useful to understand the critical point that affects HM Mozzarella cheese frozen storage and to find possible ways that can limit the degree of modifications of the matrix. Further studies would be useful to understand how cheesemaking parameters and cheese characteristics can influence the storability of HM Mozzarella cheese in frozen conditions.

6.6 Conflicts of Interest

The authors declare no potential conflicts of interest concerning the research, authorship, and/or publication of this article.

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6.10 Figures and tables

Figure 1. SDS-PAGE electrophoretogram of HM Mozzarella cheese samples (batch 1) at different times of frozen and refrigerated storage. The major proteins (β-casein (β-CN), α_s-caseins (α_s-CN, para-κ-CN) are indicated on the gel; also γ_1, γ_2, γ_3-CNs and β-CN (f69–209) (γ_4) were identified according to literature data.

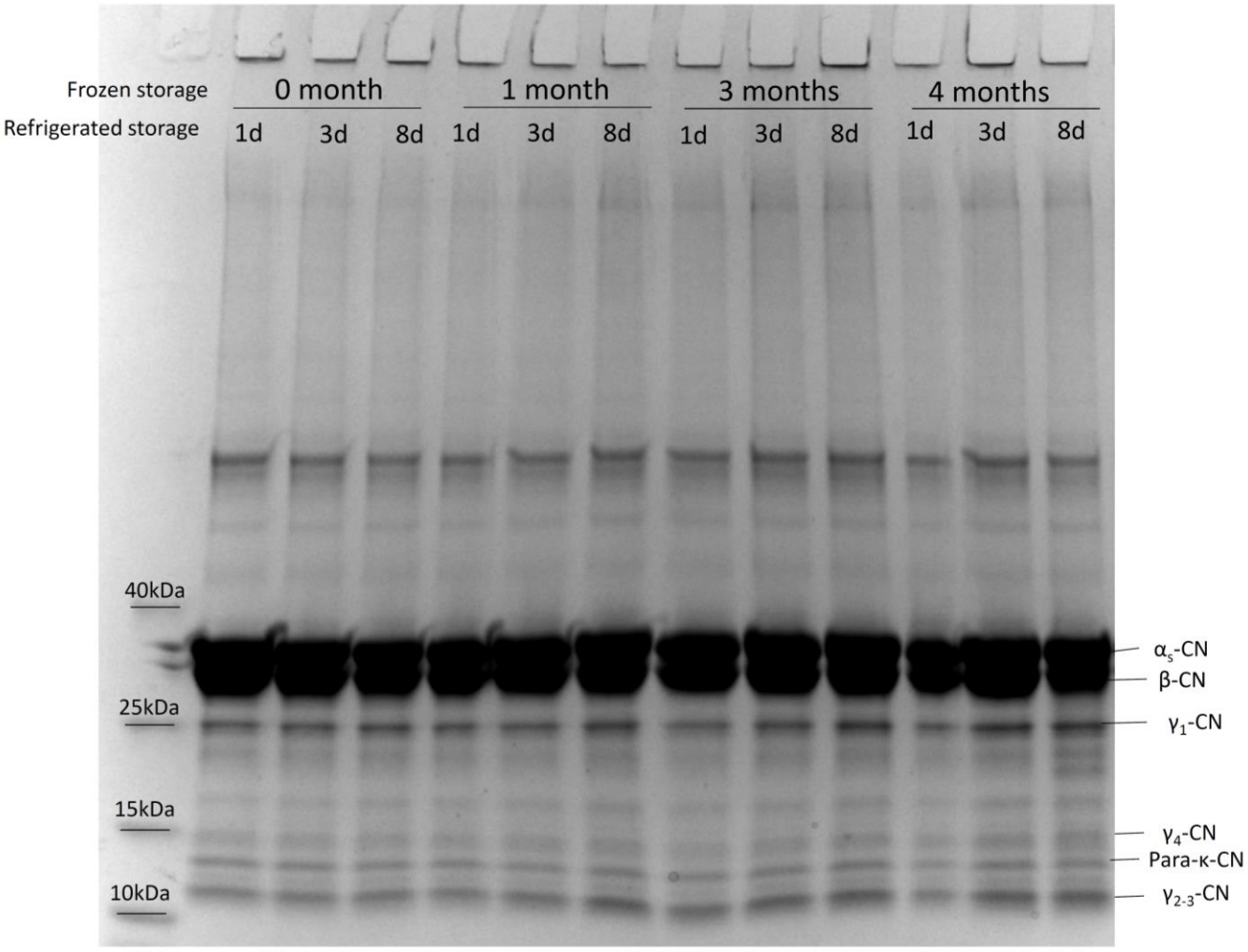
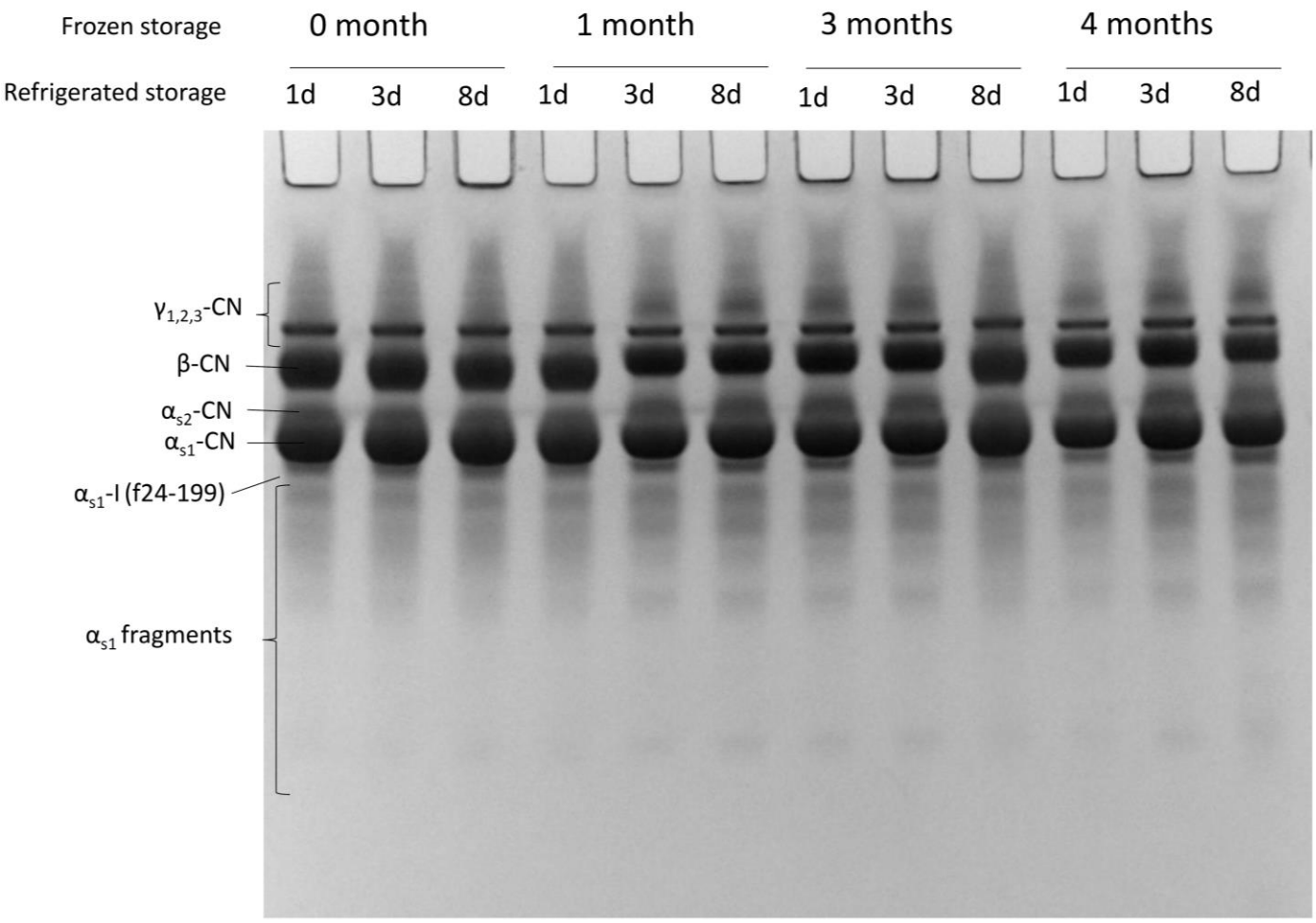
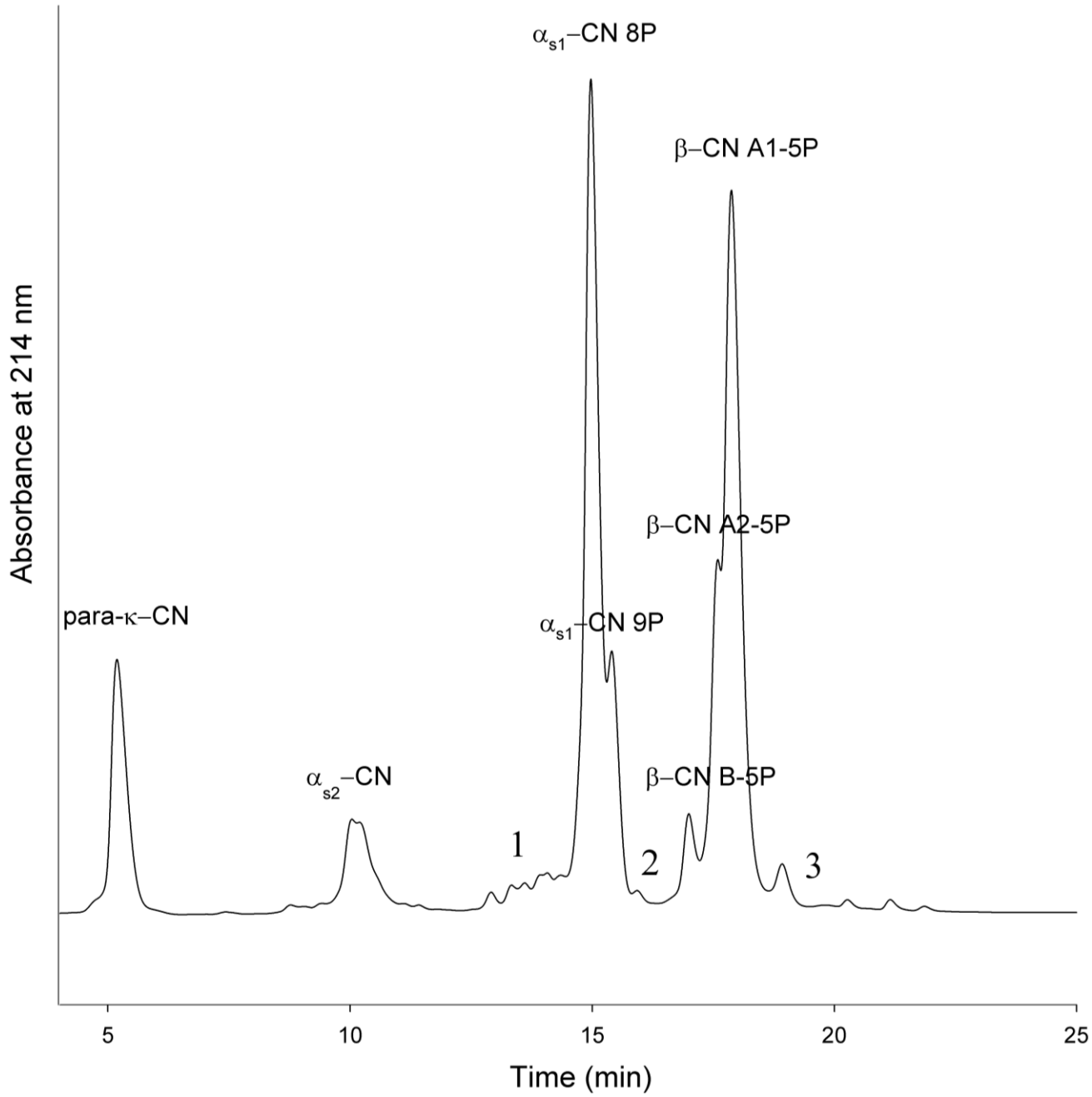


Figure 2. Urea-PAGE electrophoretogram of HM Mozzarella cheese samples (batch 1) at different times of frozen and refrigerated storage. The major proteins (β-casein (β-CN), α_{s1}-casein (α_{s1}-CN) α_{s2}-casein (α_{s2}-CN), γ_1, γ_2, γ_3-CNs, α_{s1}-I (f24-199), and α_{s1} degradation products are indicated on the gel.



12 **Figure 3.** Typical HM Mozzarella cheese reverse phase HPLC chromatograph where are reported main
13 genetic variants and isoforms of major milk caseins are reported. Peaks labeled as 1, 2, 3, are degradation
14 products of caseins.



15

Figure 4. Reverse phase HPLC chromatograms relative to HM Mozzarella cheese stored in refrigerated and/or frozen conditions. Reported samples were: fresh, non-frozen cheese samples stored for 1, 3, 8 days of refrigerated storage (0.1, 0.3, 0.8); 1-month frozen stored cheese samples subsequently stored for 1, 3 8 days of refrigerated storage (1.1, 1.3, 1.8); 3-months frozen stored cheese samples subsequently stored for 1, 3 8 days of refrigerated storage (3.1, 3.3, 3.8); 4-months frozen stored cheese samples subsequently stored for 1, 3 8 days of refrigerated storage (4.1, 4.3, 4.8).

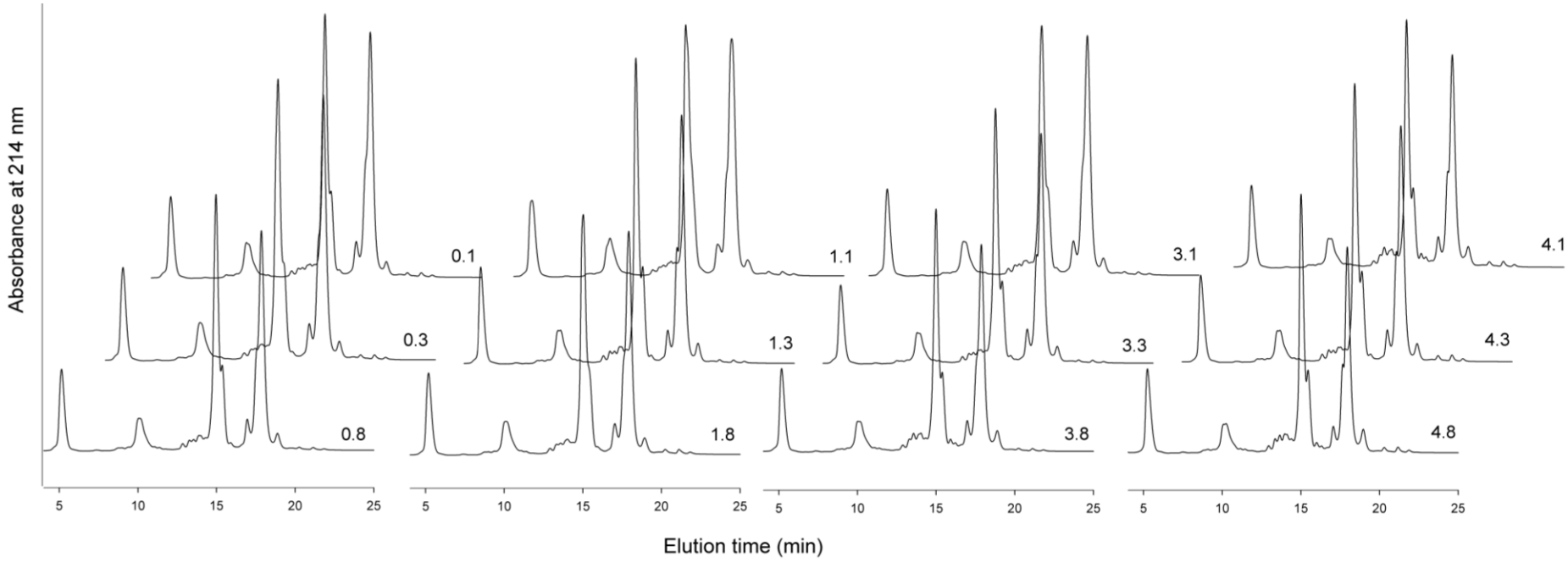
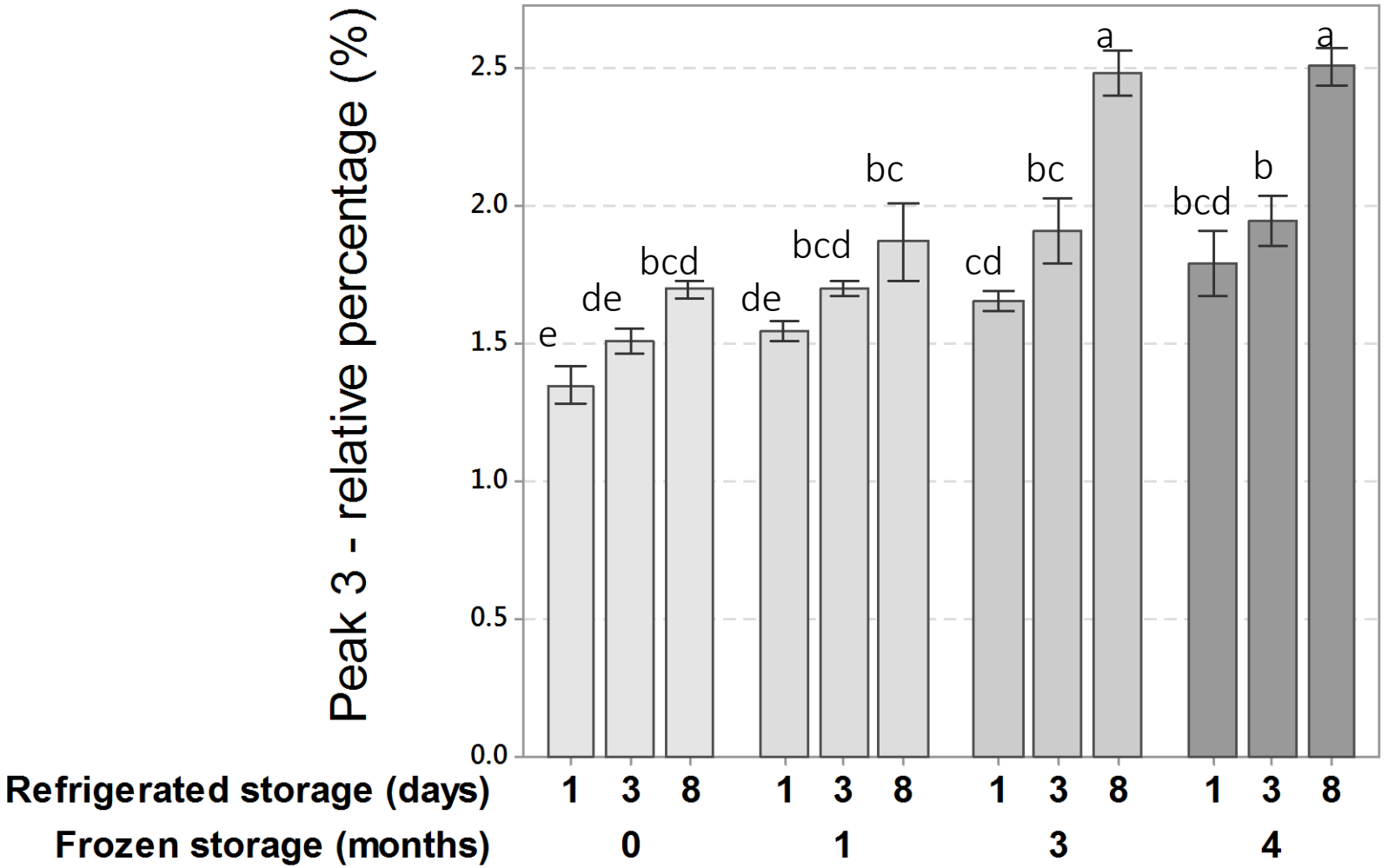


Figure 5. Peak 3 of reverse phase HPLC chromatograph, as indicated in figure 3, that correspond to a part of CNs degradation products of HM Mozzarella cheeses stored in frozen and refrigerated conditions for different times; 0-month of frozen storage represent the fresh, non-frozen cheese samples stored for 1, 3, 8 days of refrigerated storage. Different letters indicate means that are statistically different ($P<0.05$).



28 **Figure 6.** Free amino terminal estimation by fluorescamine assay as leucine equivalents (mM) in HM Mozzarella cheeses stored in frozen
29 and refrigerated conditions for different times; 0-month of frozen storage represent the fresh, non-frozen cheese samples stored for 1, 3,
30 8 days of refrigerated storage. Different letters indicate means that are statistically different (P<0.05).

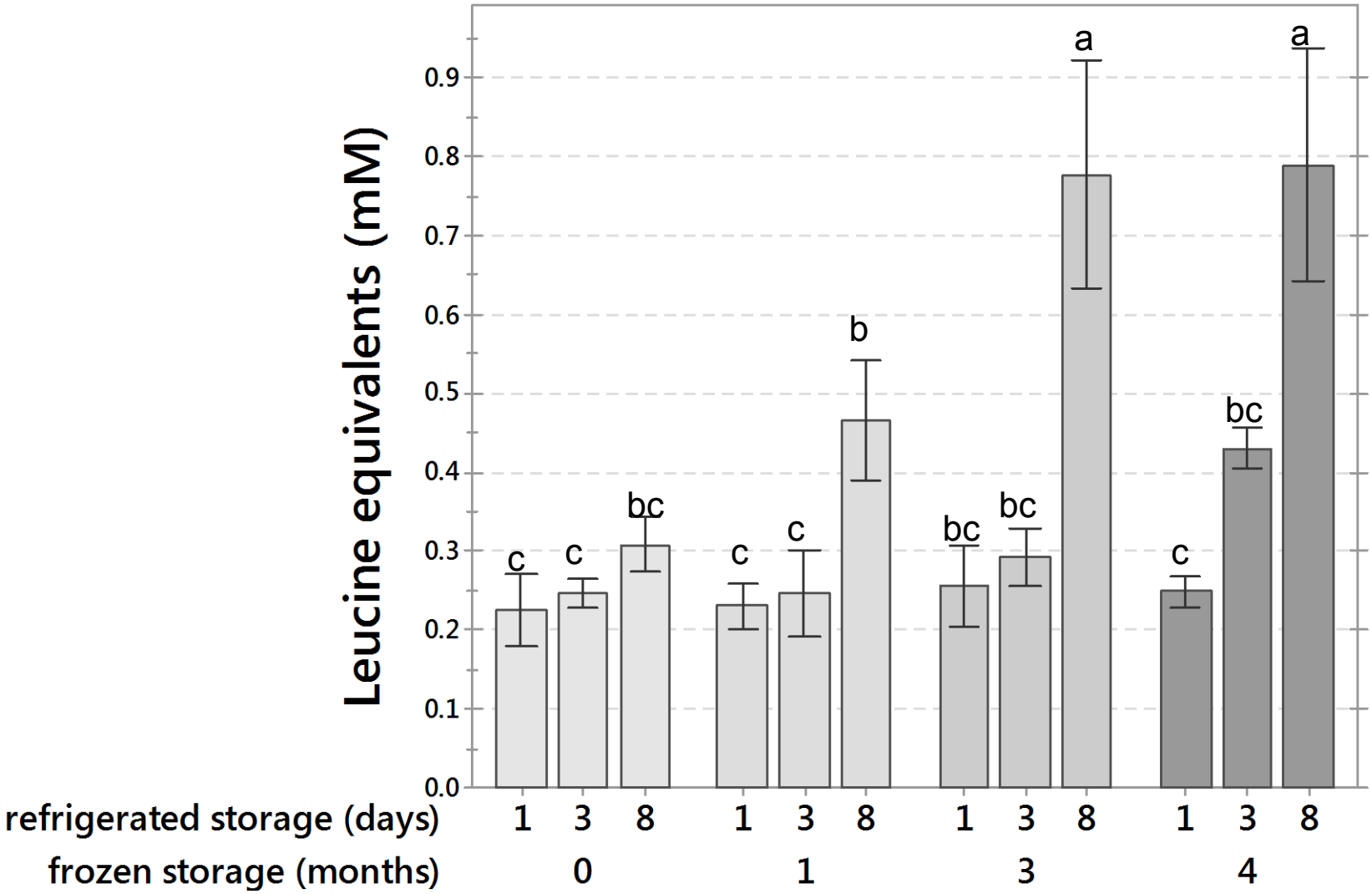


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Figure 7. ^{1}H T_1 NMR relaxation curves of HM Mozzarella cheese samples at different frozen storage (*Ft*) and refrigerated storage (*Rt*) times. Reported curves were: fresh, non-frozen cheese samples stored for 1, 3, 8 days of refrigerated storage (0.1, 0.3, 0.8); 1-month frozen stored cheese samples subsequently stored for 1, 3 8 days of refrigerated storage (1.1, 1.3, 1.8); 3-months frozen stored cheese samples subsequently stored for 1, 3 8 days of refrigerated storage (3.1, 3.3, 3.8); 4-months frozen stored cheese samples subsequently stored for 1, 3 8 days of refrigerated storage (4.1, 4.3, 4.8).

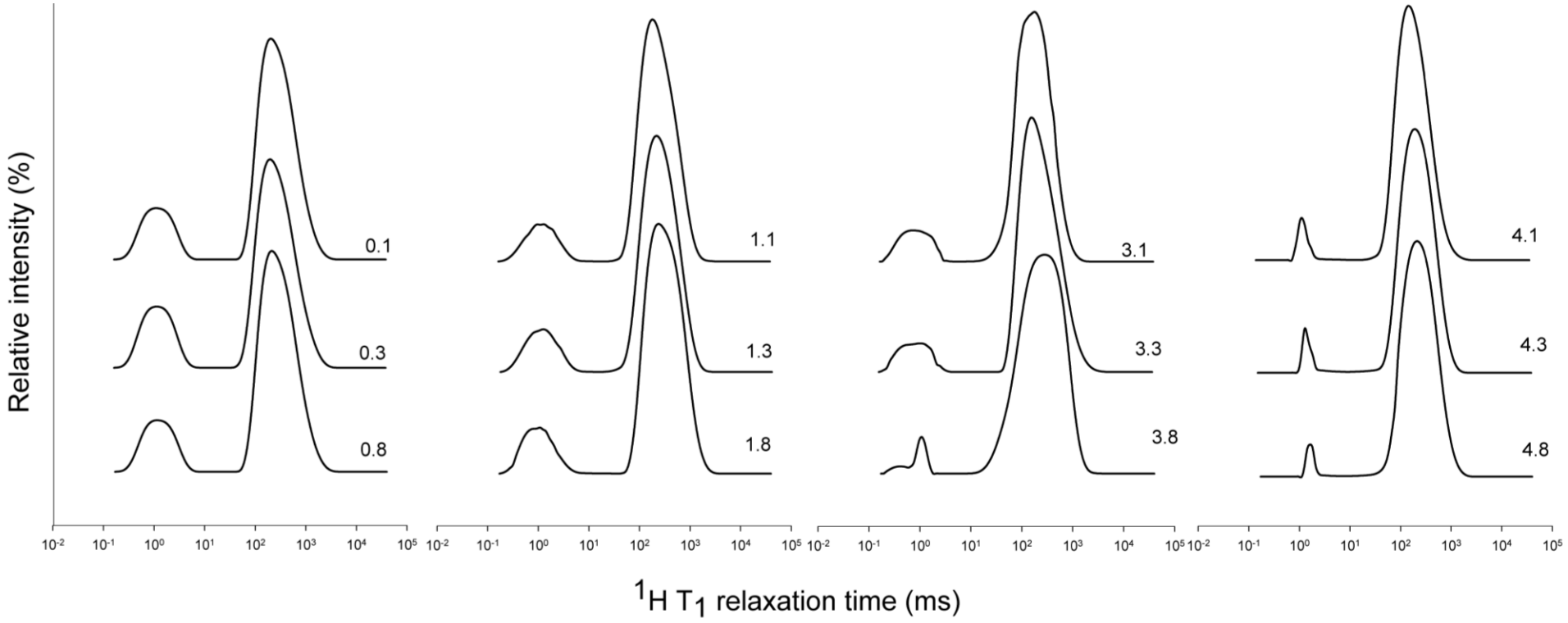


Figure 8. ^{1}H T_2 NMR relaxation curves of HM Mozzarella cheese samples at different frozen storage (*Ft*) and refrigerated storage (*Rt*) times. Reported curves were: fresh, non-frozen cheese samples stored for 1 and 8 days of refrigerated storage (0.1, 0.8, respectively); 1, 3 4 months frozen stored cheese samples subsequently stored for 8 days of refrigerated storage (1.8, 3.8, 4.8, respectively).

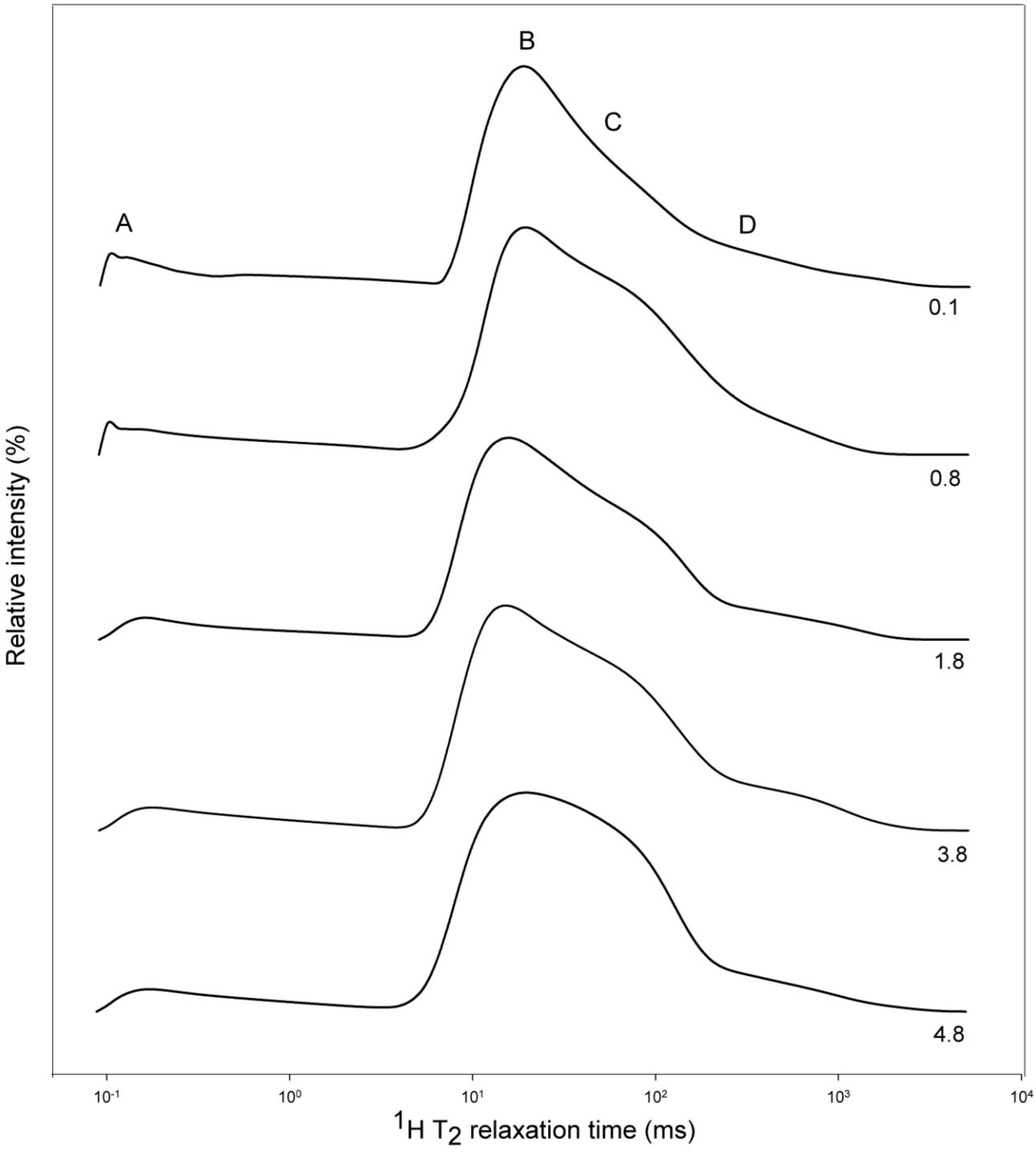


Figure 9. Variation of relative percentage of $^{1}H\ T_1$ population A of HM Mozzarella cheeses stored for different frozen storage times (*Ft*); data are reported as means of all refrigerated storage times.

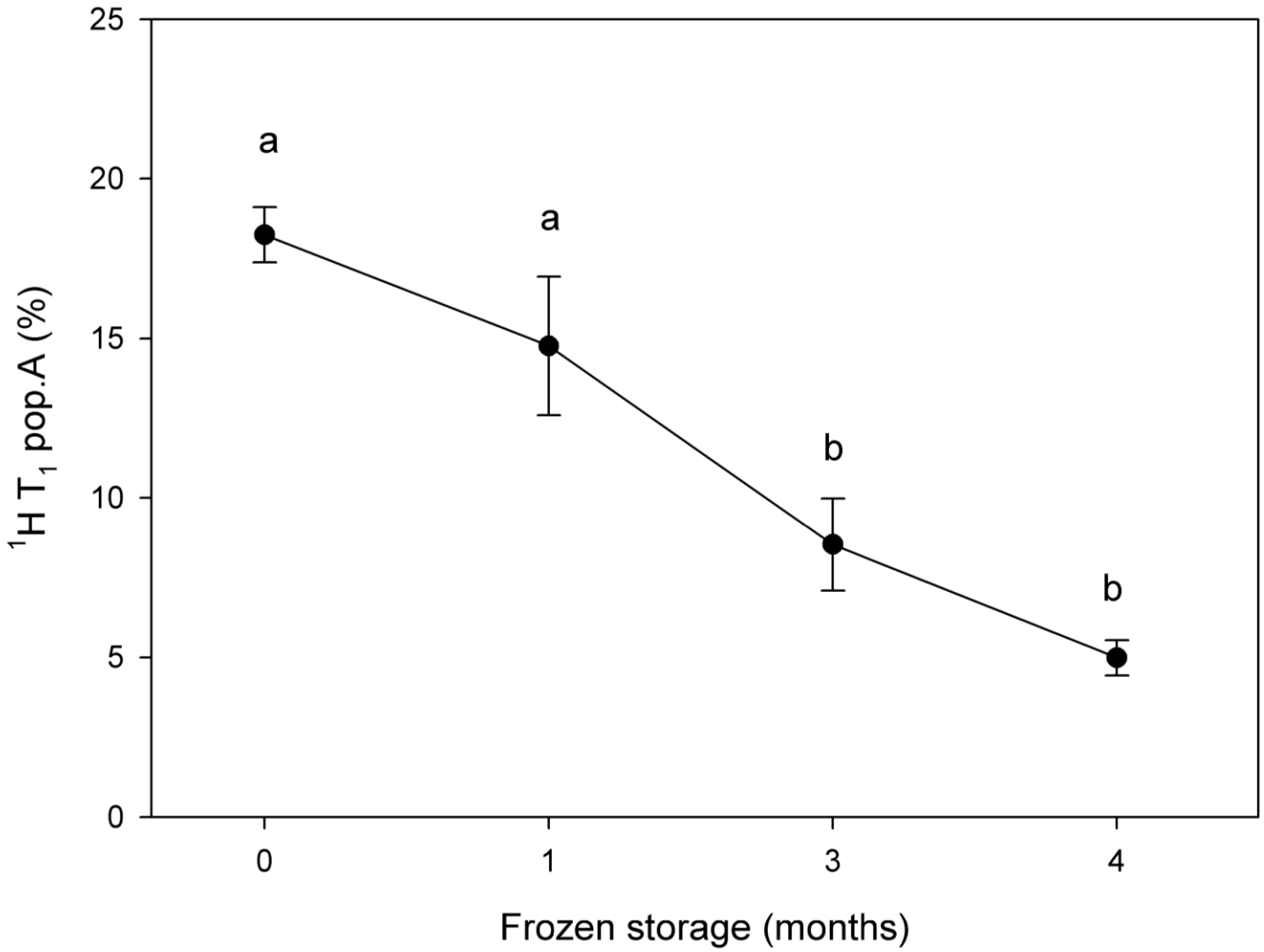


Figure 10. Variation of ^{1}H T_2 NMR variables of HM Mozzarella as a function of different frozen and refrigerated storage times (*Ft, Rt*): ^{1}H T_{2A} relaxation time (a, b), and relative percentage of populations B and C (c, d).

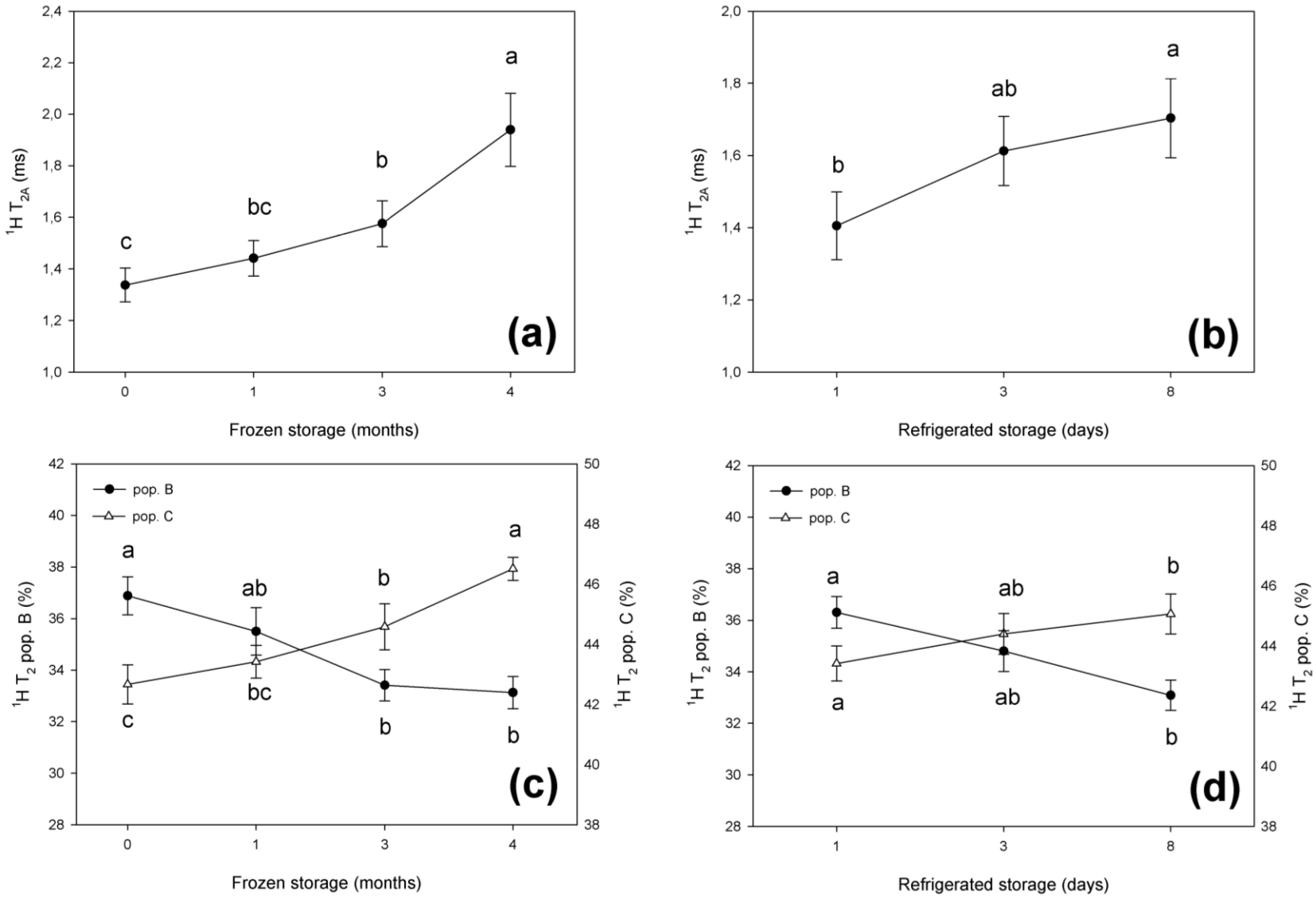
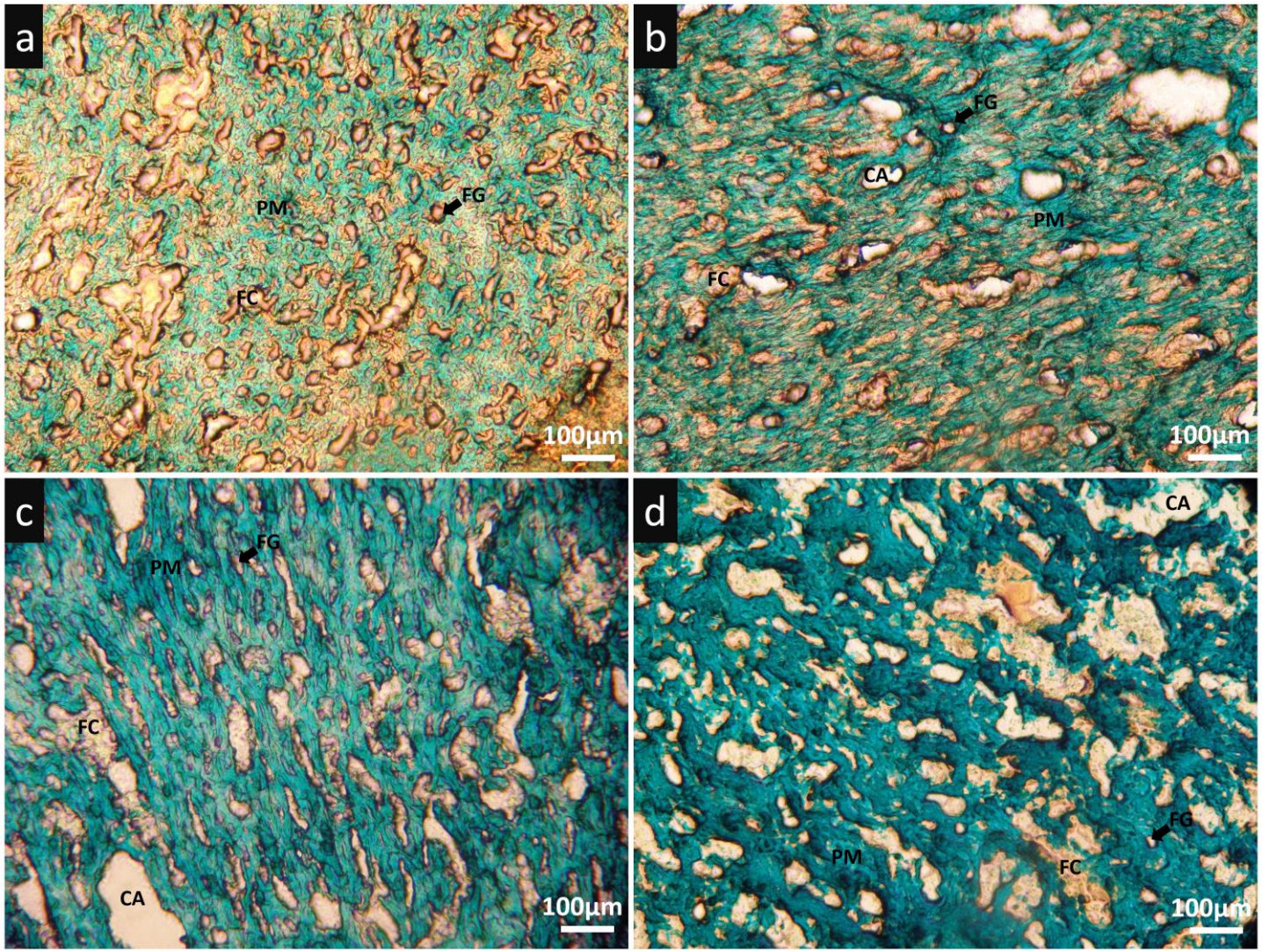


Figure 10. High moisture Mozzarella cheese microstructure observed at different frozen storage periods, 1 day after thawing: **a)** 0 month (fresh, non-frozen control cheese), (b) 1 month, (c) 3months, (d) 4 months. PM: protein matrix; FG: fat globule; FC: fat cluster; CA: serum channel.



53 **Figure 11.** Comparison between the external surface of fresh (a), and 4-months frozen stored (b) HM Mozzarella cheeses.

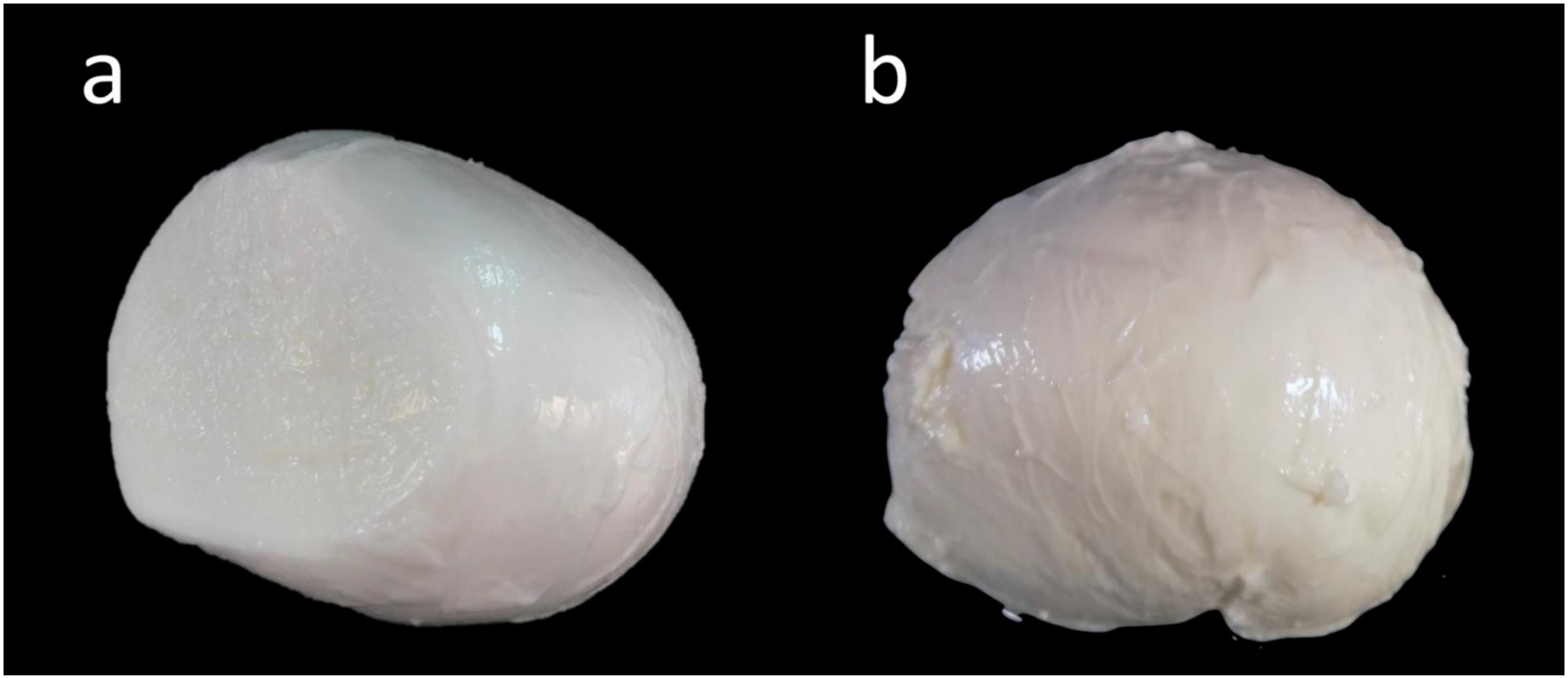


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Figure 12. Variation of appearance of surface smoothness (a), perceived oxidized (c) and bitter tastes (d) as a function of frozen storage (months) and reported as means of all refrigerated storage times; variation of sensory hardness (b) as a function of refrigerated storage (days) and reported as means of all frozen storage times. Values are expressed as score points (minimum score 0, maximum score 9) evaluated by a trained panel group (n=5).

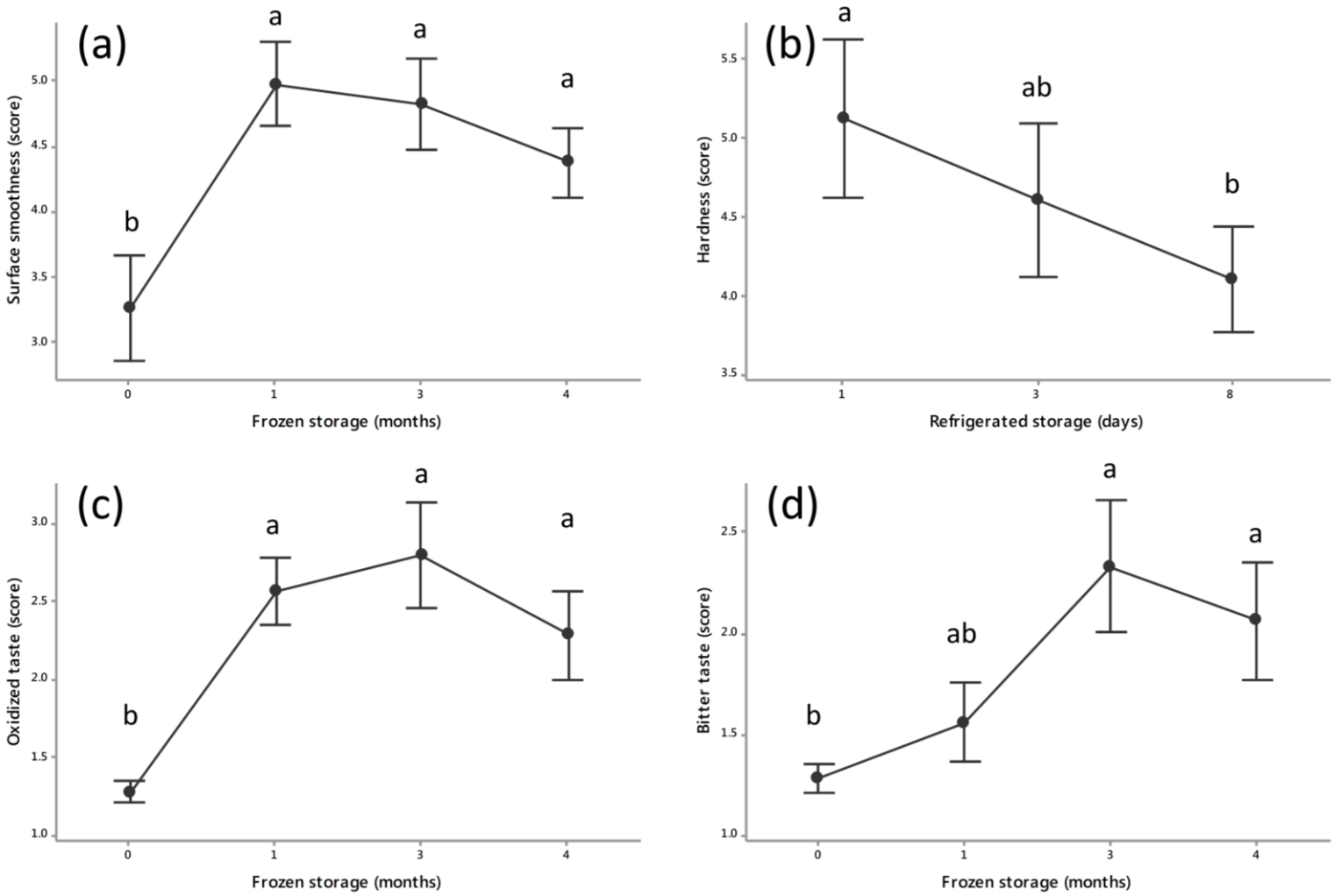

Figure 13. Principal component analysis (PCA) score (b, c) and loading plots (a). Principal components were calculated considering a reduced list of chemical, physical, rheological and sensory parameters; cheese samples were clustered according to the results obtained from the hierarchical cluster analysis based on squared Euclidean distances and Ward's method. Samples were labelled according to the different frozen storage period (b) and refrigerated storage period (c).

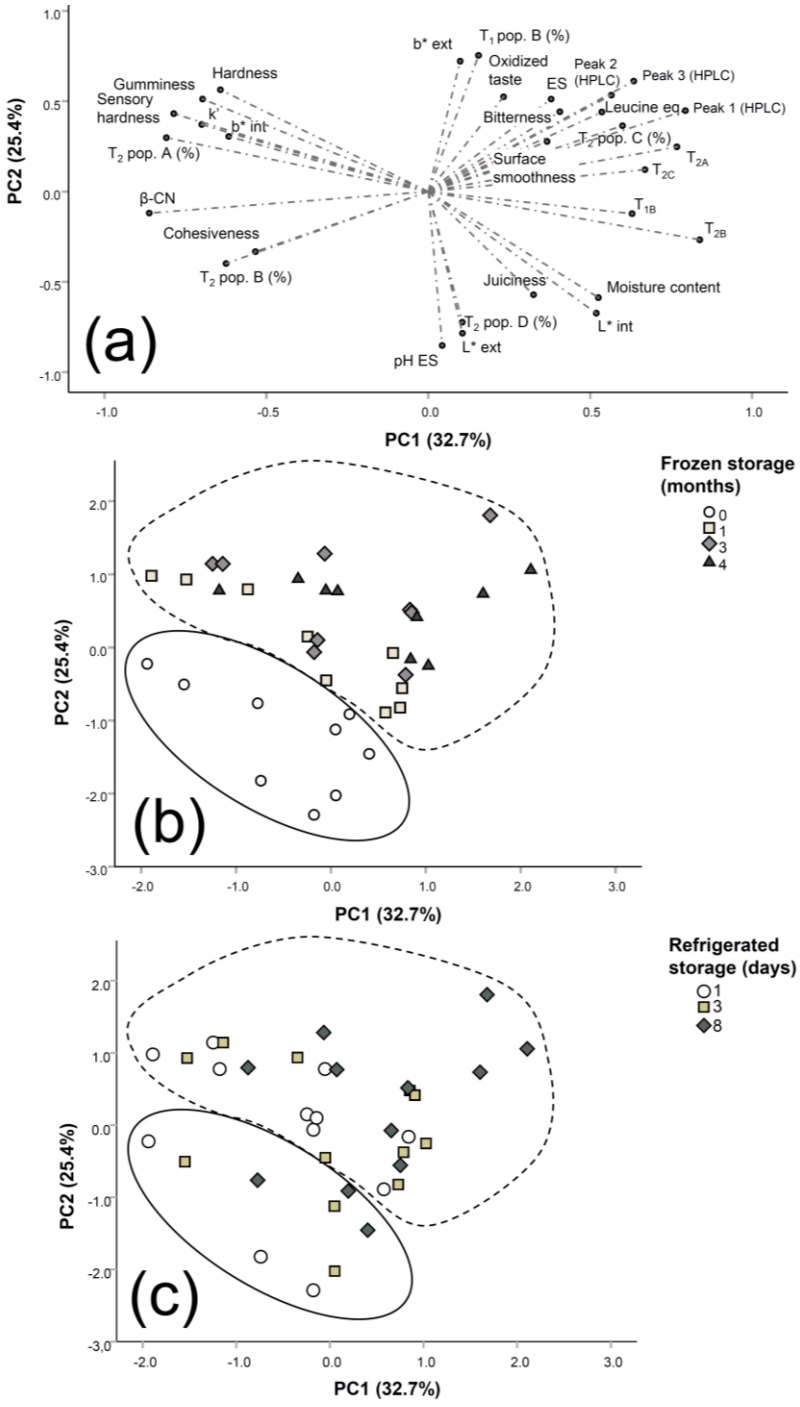

Table 1. P values obtained from split plot ANOVA models of the evaluated physical and chemical parameters considering frozen storage (*Ft*) and refrigerated storage (*Rt*) as variables. Measured parameters were the moisture content (MC), protein content (PT), fat content (FAT), expressible serum (ES), pH and electrical conductivity (COND) of expressible serum, and external and internal colorimetric coordinates (L^*ext, a^*ext, b^*ext and L^*int, a^* int, b^* int).

Parameter	MC	PR	FAT	ES	pH	COND	L^*ext	a^*ext	b^*ext	L^*int	a^*int	b^*int
Batch (*Block*)	0.006	0.030	0.049	0.129	0.300	0.016	0.126	0.010	0.649	0.001	0.019	0.001
Frozen storage (*Ft*)	0.691	0.248	0.364	0.032	0.050	0.861	0.013	0.881	0.031	0.005	0.618	0.095
B x Ft												
Refrigerated storage (*Rt*)	0.740	0.170	0.118	0.821	0.599	0.352	0.739	0.072	0.812	0.334	0.041	0.267
Ft × *Rt*	0.638	0.437	0.319	0.111	0.342	0.919	0.591	0.351	0.927	0.977	0.492	0.334

Table 2. Physical and 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. Measured parameters were the moisture (MC), protein (PT) and fat content (FAT), expressible serum (ES), pH and electrical conductivity (COND) of ES, external and internal colorimetric coordinates (L^*ext, a^*ext, b^*ext and L^*int, a^* int, b^* int).

Frozen storage (months)	MC (% w/w)	PR (% w/w)	FAT (% w/w)	ES (%)	pH (-)	COND (mS cm^{-1})	L^*ext (-)	a^*ext (-)	b^*ext (-)	L^*int (-)	a^*int (-)	b^*int (-)
0	61.08 ± 2.65	18.31 ± 1.48	20.71 ± 2.07	52.87^{c} ± 2.29	5.92^{a} ± 0.04	9.01 ± 2.48	94.14^{a} ± 0.28	0.34 ± 0.09	13.48^{b} ± 0.46	92.15^{a} ± 0.80	0.33 ± 0.12	19.09 ± 1.07
1	61.31 ± 2.62	17.51 ± 1.13	21.91 ± 1.49	56.36^{b} ± 1.89	5.82^{b} ± 0.12	8.79 ± 1.94	93.29^{b} ± 0.43	0.32 ± 0.06	14.95^{a} ± 0.52	91.45^{b} ± 0.59	0.28 ± 0.08	19.47 ± 0.65
3	60.81 ± 2.50	17.81 ± 1.21	21.75 ± 1.70	56.98^{ab} ± 2.44	5.73^{c} ± 0.06	9.62 ± 1.37	93.15^{b} ± 0.49	0.36 ± 0.14	15.42^{a} ± 1.02	91.51^{b} ± 0.43	0.35 ± 0.15	19.11 ± 0.78
4	60.19 ± 1.56	18.54 ± 1.10	19.91 ± 2.92	58.12^{a} ± 2.23	5.73^{c} ± 0.06	9.35 ± 2.14	93.42^{b} ± 0.28	0.36 ± 0.17	15.01^{a} ± 0.56	91.68^{ab} ± 0.54	0.31 ± 0.14	18.99 ± 0.78

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

Table 3. P values obtained from split plot ANOVA models of caseins, relative degradation products measured with reverse phase HPLC and of N-terminals estimation with fluorescamine assay considering frozen storage (*Ft*) and refrigerated storage (*Rt*) as variables. Peak 1, 2, 3, represent the peaks of CN degradation products observed in the samples as indicated in figure 3.

Parameter	α_{s1}-CN	α_{s2}-CN	β-CN	para-κ-CN	peak 1	peak 2	peak 3	Fluorescamine
Batch (*Block*)	0.018	0.003	0.007	0.024	0.064	0.461	0.283	0.221
Frozen storage (*Ft*)	0.283	0.149	0.135	0.335	0.024	0.300	0.003	0.012
B x *Ft*								
Refrigerated storage (*Rt*)	0.917	0.077	<0.001	0.173	<0.001	0.009	<0.001	<0.001
Ft × *Rt*	0.790	0.480	0.180	0.934	0.771	0.500	0.021	0.043

Table 4. Relative percentage of β-casein, α_{s1}-casein (α_{s1}-CN), their relative degradation products and fluorescamine results of fresh HM Mozzarella cheeses (0 month of frozen storage) and cheeses having 1, 3 and 4 months of frozen storage before thawing and refrigerated storage period (1, 3, 8days). Peaks 1, 2, 3, represent the relative percentage of CN degradation products observed in the samples as indicated in figure 3.

Frozen storage (months)	Refrigerated storage (days)	β-CN (%)	peak 1 (%)	peak 2 (%)	peak 3 (%)	Fluorescamine (eq. leucine mM)
0	1	$40.28^{a} \pm 0.54$	$3.73^{d} \pm 0.17$	$0.31^{b} \pm 0.06$	$1.35^{e} \pm 0.17$	$0.23^{c} \pm 0.08$
	3	$38.89^{bcd} \pm 0.39$	$4.06^{cd} \pm 0.18$	$0.35^{ab} \pm 0.01$	$1.51^{de} \pm 0.09$	$0.25^{c} \pm 0.03$
	8	$37.91^{edf} \pm 0.47$	$4.38^{bcd} \pm 0.14$	$0.39^{ab} \pm 0.02$	$1.70^{bcd} \pm 0.08$	$0.31^{cb} \pm 0.06$
1	1	$39.82^{ab} \pm 1.35$	$3.98^{cd} \pm 0.22$	$0.38^{ab} \pm 0.01$	$1.55^{de} \pm 0.03$	$0.23^{c} \pm 0.05$
	3	$38.81^{bcd} \pm 1.20$	$4.31^{cd} \pm 0.30$	$0.42^{ab} \pm 0.02$	$1.68^{bcd} \pm 0.08$	$0.25^{c} \pm 0.09$
	8	$37.59^{ef} \pm 1.30$	$4.63^{abcd} \pm 0.40$	$0.45^{ab} \pm 0.05$	$1.84^{bc} \pm 0.15$	$0.47^{b} \pm 0.13$
3	1	$39.35^{abc} \pm 1.07$	$4.29^{cd} \pm 0.24$	$0.38^{ab} \pm 0.02$	$1.66^{cd} \pm 0.06$	$0.26^{cb} \pm 0.09$
	3	$38.53^{bcd} \pm 1.29$	$4.58^{abcd} \pm 0.71$	$0.55^{ab} \pm 0.21$	$1.91^{bc} \pm 0.32$	$0.29^{cb} \pm 0.06$
	8	$36.93^{gf} \pm 0.80$	$5.26^{ab} \pm 0.98$	$0.73^{a} \pm 0.43$	$2.65^{a} \pm 0.33$	$0.78^{a} \pm 0.25$
4	1	$38.89^{bcd} \pm 0.69$	$4.57^{bcd} \pm 0.25$	$0.42^{ab} \pm 0.02$	$1.79^{bcd} \pm 0.25$	$0.25^{c} \pm 0.03$
	3	$38.55^{bcd} \pm 1.18$	$4.72^{abc} \pm 0.29$	$0.48^{ab} \pm 0.02$	$1.95^{b} \pm 0.23$	$0.43^{cb} \pm 0.05$
	8	$36.33^{g} \pm 1.66$	$5.50^{a} \pm 0.48$	$0.60^{a} \pm 0.15$	$2.51^{a} \pm 0.18$	$0.79^{a} \pm 0.25$

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

Table 5. P values obtained from split plot ANOVA models of ^{1}H T_1, T_2 NMR variables of HM Mozzarella cheeses, in relations frozen storage (*Ft*) and refrigerated storage (*Rt*). Measured variables were: T_1 relaxation times (T_{1A}, T_{1B}) and relative percentages (T_1 popA, T_1 popB) of two ^{1}H populations; T_2 relaxation times (T_{2A}, T_{2B}, T_{2C}, T_{2D}) and relative percentages (T_2 popA, T_2 popB, T_2 popC, T_2 popD) of four ^{1}H populations.

Parameter	T_1 popA (%)	T_1 popB (%)	T_{1A}	T_{1B}	T_2 popA (%)	T_2 popB (%)	T_2 popC (%)	T_2 popD (%)	T_{2A}	T_{2B}	T_{2C}	T_{2D}
Batch (*Block*)	0.411	0.411	0.526	0.005	0.002	0.663	0.063	0.348	0.081	0.004	0.194	0.417
Frozen storage (*Ft*)	0.014	0.014	0.022	0.882	0.580	0.029	0.013	0.134	0.019	0.714	0.449	0.906
B x *Ft*												
Refrigerated storage (*Rt*)	0.588	0.588	0.129	0.028	0.479	0.003	0.030	0.952	0.012	0.085	0.007	0.842
Ft × *Rt*	0.826	0.826	0.639	0.824	0.806	0.998	0.299	0.669	0.711	0.282	0.423	0.251

Table 6. P values obtained from split plot ANOVA models of the evaluated rheological, textural and sensory parameters for frozen storage (*Ft*) and refrigerated storage (*Rt*). Measured parameters were power law regression parameters from storage modulus (k', n'), loss modulus (k'', n'') and complex viscosity (k^*, n^*) derived from frequency sweep measurements; hardness (HAR TPA), cohesiveness (COH), gumminess (GUM), springiness (SPR) measured with TPA; perceived whiteness (WHI), translucency (TRA), hardness (HAR sens), juiciness (JUI), paste and surface smoothness (PAS-SMO, SUR-SMO), acidity (ACI), saltiness (SAL), oxidized (OXI) and bitter tastes (BIT) from sensory evaluation.

Parameter	k'	k''	k^*	n'	n''	n^*	HAR TPA	COH	GUM	SPR	WHI	TRA	HAR sens	JUI	PAS-SMO	SUR-SMO	ACI	SAL	OXI	BIT
Batch (*Block*)	0.028	0.025	0.032	0.654	0.700	0.364	0.001	0.212	0.001	0.032	0.242	0.198	0.001	0.027	0.843	0.026	0.951	0.437	0.141	0.318
Frozen storage (*Ft*)	0.922	0.939	0.894	0.241	0.392	0.219	0.005	0.045	0.013	0.361	0.199	0.147	0.249	0.363	0.643	0.012	0.346	0.201	0.021	0.036
B x *Ft*																				
Refrigerated storage (*Rt*)	0.216	0.191	0.267	0.250	0.823	0.930	0.414	0.168	0.206	0.768	0.515	0.931	0.046	0.900	0.770	0.475	0.353	0.680	0.800	0.533
Ft × *Rt*	0.826	0.801	0.765	0.658	0.532	0.620	0.041	0.067	0.123	0.860	0.439	0.057	0.744	0.184	0.018	0.134	0.356	0.564	0.741	0.55

Table 7. Texture profile analysis (hardness, cohesiveness, gumminess, springiness) and rheological parameters derived from frequency sweeps fitted using power law regression equations (k', k'', k*, n', n'', n*) of fresh HM Mozzarella cheeses (0 month of frozen storage) and cheeses having 1, 3 and 4 months of frozen storage before thawing; data are reported as means of all refrigerated storage times.

Frozen storage (months)	Hardness (N)	Gumminess (N)	Cohesiveness (-)	Springiness (-)	k' (Pa s)	k'' (Pa s)	k* (Pa s)	n' (-)	n'' (-)	n* (-)
0	19.07^{c} ± 7.17	11.76^{c} ± 4.48	0.62^{a} ± 0.03	0.73 ± 0.04	15171 ± 6633	4791 ± 2059	2590 ± 1157	0.166 ± 0.009	0.162 ± 0.012	0.166 ± 0.009
1	23.94^{b} ± 7.73	14.13^{b} ± 4.77	0.59^{b} ± 0.02	0.71 ± 0.04	13786 ± 5364	4415 ± 1729	2324 ± 887	0.172 ± 0.008	0.164 ± 0.007	0.172 ± 0.008
3	26.26^{a} ± 5.57	15.35^{a} ± 3.66	0.58^{b} ± 0.02	0.72 ± 0.04	13800 ± 1727	4398 ± 546	2315 ± 292	0.177 ± 0.007	0.17 ± 0.004	0.182 ± 0.019
4	19.68^{c} ± 5.19	11.42^{c} ± 3.09	0.58^{b} ± 0.01	0.75 ± 0.06	13483 ± 4099	4302 ± 1332	2253 ± 686	0.177 ± 0.016	0.169 ± 0.014	0.176 ± 0.016

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

Table 8. P values obtained from split plot ANOVA models of the evaluated sensory parameters for the factors considered: frozen storage (*Ft*) and refrigerated storage (*Rt*). Evaluated sensory parameters were perceived whiteness (WHI), translucency (TRA), hardness (HAR sens), juiciness (JUI), paste and surface smoothness (PAS-SMO, SUR-SMO), acidity (ACI), saltiness (SAL), perception of oxidized (OXI) and bitterness (BIT).

Parameter	WHI	TRA	HAR sens	JUI	PAS-SMO	SUR-SMO	ACI	SAL	OXI	BIT
Batch (*Block*)	0.242	0.198	0.020	0.027	0.843	0.034	0.951	0.437	0.138	0.318
Frozen storage (*Ft*)	0.200	0.147	0.249	0.363	0.643	0.010	0.346	0.201	0.126	0.036
B x *Ft*										
Refrigerated storage (*Rt*)	0.515	0.931	0.045	0.899	0.138	0.475	0.353	0.680	0.789	0.533
Ft × *Rt*	0.439	0.056	0.744	0.184	0.359	0.134	0.356	0.564	0.648	0.550

7. Application of NIR spectroscopy and image analysis for the characterization of grated Parmigiano-Reggiano cheese

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7.1 Abstract

Grated Parmigiano–Reggiano cheese holds a valuable market segment and its quality strictly depends on the amount of rind, size, shape of cheese particles and original cheese properties. Textural properties of rind and inner part of the cheese significantly affect size and shape of grated particles. Rind produces a higher amount of finer and less circular particles than the inner region. Rind content established by European regulation (maximum 18%) is a major issue and could be successfully predicted by multivariate models developed on Near-infrared (NIR) spectra. Image Analysis (IA) was a suitable method to estimate rind percentage that was found positively correlated to number of particles, total surface covered by particles and circularity. IA and NIR spectroscopy enabled to characterize the distribution of the particle in dimensional classes and could be used to control the maximum limit of 25% of particles finer than 0.5mm provided by European regulation.

Keywords: Parmigiano-Reggiano; Grated cheese; Cheese rind; Product compliance; NIR spectroscopy; Image Analysis; Particle size; Partial Least Squares regression

7.2 Introduction

Cheese rind in long-ripened cheese has different chemical and physical characteristics than the other parts of the cheese, as it is exposed during ripening to environmental conditions (i.e. air, water vapor pressure, light; surface microorganisms, sodium chloride), that contribute to differently modify the initial properties of the cheese matrix. The more evident changes for long-ripened cheeses without a specific surface microbiota are the decrease of moisture content and water activity, and the consequent decrease of proteolytic activities operated by indigenous proteinases (Cattaneo et al., 2008; Mayer, 1996). Furthermore, the prolonged contact with air and light increase the degree of oxidation (Karoui, Dufour, & De Baerdemaeker, 2007). These phenomena can impact on the sensory perception of the rind, that is generally characterized by different sensorial properties than the inner part of the cheese, as evidenced by the presence of odor and flavor of "rind" among the descriptors of cheese sensory attributes (Biasioli et al 2006; Zanoni, 2010). Industrially, hard long-ripened cheese wheels are brushed and/or washed and then are either sliced to be sold in portions with or without rind or are addressed to the grater. The market of cheese portions without rind created the availability of large amount of rind with a lower value that can be sold separately as is or can be grated with other trimmed pieces of cheese and/or whole wheels As the presence of excessive amounts of rind in grated cheese can be perceivable by the consumer and can have a negative impact on the sensory characteristics (Zanoni & Hunter, 2015), the quality of grated cheese depends both on the original properties of the whole cheese and on the percentage of rind. In this context, some grated cheese produced under the rules of European Protected Designation of Origin (**PDO**) must comply with specific limitation regarding the presence and percentage of rind (e.g. Grana Padano, Parmigiano Reggiano, Pecorino Sardo) (DOOR, 2018). Parmigiano–Reggiano (**P–R**) is a cooked, long-ripened, hard cheese made in Northern Italy registered as a PDO in European Union (European Regulation No. 1151/2012). The PDO status of P-R cheese is extended to the grated type, with grating and packaging operations that must take place in the same area of origin as that of production. Grated P-R cheese accounted for 13.5% of the overall market of P-R cheese in 2017 (+5.2% compared to 2016) and this percentage continuously increased in the last years (Parmigiano-Reggiano Consortium, 2017). PDO designation is restricted to grated cheeses accounting specific technical parameters, such as minimum 12 months of ripening, fat content not less than 32% in proportion to dry matter and moisture between 25-35%. Additionally, the aspect should be homogeneous, with less than 25% (without specifying if % is in weight or in volume) of particles having diameter less than 0.5 mm, and a quantity of rind less than 18% (w/w); rind is defined by the regulation as the external part of the

cheese with a depth of 6 mm (DOOR, 2018). To date, no official analytical methods for the quantification of rind and finer particles are considered by the PDO regulation. The limit of 18% of rind in grated cheese can be surpassed because of process issues (e.g. mixing errors before grating when both whole wheels and trimmed parts are used or during packaging operations) or because of fraudulent reasons. Particle size properties are an important issue in the quality control of food industries since they can affect texture, mouthfeel and further processing of products. Main techniques employed to measure particle size range from traditional sieving to sedimentation and light scattering; to this purpose, the use of Image Analysis (IA) has proven to be a valid alternative (Brosnan & Sun, 2004; Caccamo et al., 2004; Febbi, Menesatti, Costa, Pari, & Cecchini, 2015; Iezzi et al., 2012; Sugimoto, Hashimoto, Fukuike, Kodama, & Minagi, 2014), also thanks to the evolution and diffusion of high resolution digital machines. In particular, IA measurements have also been proposed to measure particle size properties of shredded cheese samples (Ni & Guansekaran, 2004). Studies in literature reported also the relation between product's hardness and particle size resulting from wheat milling processes (Fang, C., & Campbell, 2003; Campbell et al., 2007). On the contrary, no literature data was found on the relation between textural and particle size properties of grated cheese. Concerning the detection of uncontrolled amount of rind in long ripened grated cheeses, analytical procedures range from chemical methods (Cattaneo et al., 2008) to NMR techniques (Shintu & Caldarelli, 2005), capacitive techniques (Cevoli et al., 2015), waveguide spectroscopy (Cevoli, Ragni, Gori, Berardinelli, & Caboni, 2012) and Near-infrared spectroscopy (NIRs) approaches (Barzaghi et al., 2016; Cevoli, Fabbri, Gori, Caboni, & Guarnieri, 2013; Cevoli, Gori, Nocetti, Cuibus, Caboni, & Fabbri, 2013; Musi & Filippi, 2015). NIRs has also been applied as a good and fast alternative for particle size measurements of lactose monohydrate particles and wheat flour (Frake et al., 1998; Zhu, Xing, Lu, Huang, & Ng, 2017). Among other several analytical approaches in food science, NIRs offers an interesting option of investigation: it does not require solvents or sample preparation and the same sample can be employed for other analytical tests. Moreover, the performance of diffuse reflectance analysis could be improved by using an integrating sphere in case of not homogeneous solid foods, especially when the sample is reduced in powder (Subramanian, & Rodriguez-Saona 2009). Overall costs are low and analysis times are very short if compared with traditional analytical approaches. Considering the limitation imposed by PDO regulation and the risk of quality loss when an excessive amount of rind is present in the grated product, P-R dairies need fast and reliable methods to control their process conditions and to verify that grated cheese comply with both legal limits and with their quality programs. The aims of this work were to study how differences of texture

and moisture content of P-R cheese can affect the particle size of grated cheese and to set up rapid methods based on IA and NIRs techniques to measure the particle size and the quantity of rind present in grated P-R cheese.

7.3 Materials and Methods

7.3.1 Sample collection and preparation

Cheese samples were collected from 3 cheese plants producing PDO Parmigiano-Reggiano (P-R) and supplied by Nuova Castelli Spa (Reggio Emilia, Italy), in portions of about 1 kg, packaged under vacuum and stored at 4° C until use. Samples were chosen among 5 cheese batches with 5 different ripening times (12, 18, 21, 24 and 30 months) to represent the variability of available P-R cheeses processed by the grater. Portions of cheese to be analyzed by texture analysis and moisture content did not need further preparation and were stored as a whole. For the preparation of grated samples, the rind of each cheese was cut at 6 mm of depth, according to the definition of rind provided by the PDO regulation and it was kept separately; the section between 6 mm and 20 mm of depth was removed, whereas the inner part of the cheese was taken as a whole. The rind and the cheese were separately grated by means of an electrically driven grater (Ardes AR7300, Milan, Italy) at Centro di Ricerca Zootecnia e Acquacoltura (CREA-ZA, Lodi, Italy). Pure grated inner part and rind samples were also analyzed by IA to find out possible relations with textural properties and moisture content. In order to prepare samples to be analyzed with NIRs and Image Analysis (IA), various mixtures of grated rind and grated inner part of cheeses were combined in different proportions and then accurately manually mixed into plastic bags. To simulate the wide variability occurring in industrial plants where whole wheels or pieces of cheese with different ages (rind and cheese) are grated, samples were combined at different ratios. The presence of different amounts of rind was set from 0 to 36% w/w for a total of 13 different percentages of rind (Table 1). Samples were stored at 4°C. Totally, 131 samples were analyzed by NIRs for the determination of the percentage of rind. Out of the 131 samples of the entire dataset, 50 samples were analyzed by IA to build-up a Partial Least Square (PLS) model for the quantification of cheese rind based on particle size measurements and a model for the determination of particle size properties based on NIR data.

7.3.2 Application of NIRs in a P-R cheese grating industry

The best built-up NIR-PLS model was employed to evaluate the content of rind detected during filling and packaging operations at an industrial scale. P-R cheeses ripened 28 ± 2 months were grated by means of an industrial grater (Model HP 20, Cavecchi Srl, Reggio Emilia, Italy) to produce totally seven batches of grated cheese: each batch was monitored at six fixed production times (T0, T1, T2, T3, T4 and T5 min), collecting a package of 200 g of grated cheese from the packaging line. Cheese batches to be grated were composed by trimmed pieces of cheese of various dimension. Before processing, the grating system was cleaned to remove any residue derived from the previous batch. The collected samples were stored at 4°C until they were analyzed. Results are expressed as the mean of 4 measurements.

7.3.3 Analytical Methods

7.3.3.1 NIR Spectra acquisition of grated cheese samples

NIR spectra were acquired by a FT-NIR Tango spectrometer (Bruker, MA, USA) in the spectral range of 1000 – 2500 nm, recording 32 scans acquired with a resolution of 8 cm^{-1} as reported by Barzaghi et al. (2016). Samples were equilibrated at 25±1°C for about 1 h before analysis, mixed thoroughly and an amount of 25 g was loaded into a glass Petri plate having 95 mm of diameter. Each sample was analyzed in duplicate. Additionally, each sample was subjected to a further grinding (treated samples) by means of a blender (Osterizer model 890-48H) for 30 s, and then reanalyzed in duplicate to increase model robustness against light scattering phenomena. To develop models for rind quantification, both spectra of untreated and treated samples were considered and averaged. To create predictive models based on particle size parameters, only spectra of untreated samples were considered and averaged.

7.3.3.2 Image analysis

Images of grated cheese samples (0.21 x 0.30 m A4 scanner size) were acquired using a Hewlett Packard Scanjet 8200 scanner (Palo Alto, CA, USA) with a resolution of 600 dpi (corresponding to 236 pixels cm^{-1}) and saved in TIFF format. To avoid saturation effects and overlapping of particles, 0.3 g of grated cheese was preliminary tested as a suitable amount of sample to be carefully spread on a transparent paper to achieve a homogenous distribution. A black colored background was used to enhance contrast of acquired images; a graph paper was always included during image acquisition to set the scale, expressed in mm. Elaboration of the images was performed by using ImageJ software (National Institutes of Health, Maryland, USA); images were first turned into greyscale (8 bit) and then segmented into binary images using default algorithm, imposing threshold limits of 20 and 255 for the black background. Blanks of transparent paper (without samples) were also analyzed and to avoid the interference of dusts, the command "Open" that performs an erosion operation followed by dilation, was applied to remove pixels of undefined objects not related to cheese particles. The following parameters were taken into account: total number of particles (**n**) and total surface covered by particles (S_T) by 0.3 g of sample, circularity (**C**), Feret diameter (**F**) and minimum Feret diameter (**mF**). Feret diameter is defined as the diameter of the object connecting perpendicularly two parallel tangential lines restricting the object; it represents the diameter of the object along a specified direction. F and mF are often used as the dimensions of the particles (Febbi, Menesatti, Costa, Pari, & Cecchini, 2015). In order to study the dimensional frequency distribution of the particles, 4 size classes were identified based on F and mF diameters, namely $F_{(<0.5mm,\ 0.5\text{-}1mm,\ 1\text{-}2mm,\ >2mm)}$ and $mF_{(<0.5mm,\ 0.5\text{-}1mm,\ 1\text{-}2mm,\ >2mm)}$. Each sample was analyzed in triplicate.

7.3.3.3 Textural analysis

The textural properties of P-R cheese were measured according to the puncture test proposed by Breuil, & Meullenet (2001) using a TA.XTplus Texture Analyzer (Stable Micro Systems, Godalming, UK) equipped with a 30 kg load cell and a stainless steel cylindrical probe with a 3 mm diameter (SMS P/3, Stable Micro Systems). Measurements were conducted at 0.8 mm/s with a trigger force of 5 g until a 10 mm distance was reached; pre-test and post-test speeds were set at 1 mm/s. Hardness at rupture (H_r), distance at rupture (D_r) and initial slope (S_{ini}) were respectively defined as the positive force value (N) and the distance (mm) at which sample showed rupture of its surface (the first peak originated during the puncture cycle) and the slope of the tangent until 0.5 s from the beginning of

the analysis. Maximum hardness (H_{max}), distance at maximum force (D_{max}), positive work area (A_p) were respectively defined as the maximum positive force (N), distance to reach maximum force (mm) and the total positive work (N·mm); Mod_1 was defined as the modulus (unitless) of the vector between the origin and the maximum force of the puncture graph. Texture was evaluated in different zones of the cheese wheel, namely inner part, underrind (portion of cheese between 6 mm and 20 mm of depth from the outer part of the wheel), and rind of the cheeses at different aging times. Puncture tests of rind section exhibited H_r and D_r that coincided with H_{max} and D_{max} for all samples. Samples were equilibrated at 25°C for 24h prior to be analyzed and reported measures were averages of six replicates.

7.3.3.4 Moisture content

Moisture content of cheeses was measured by drying samples at 102°C (AOAC 926.08, 1990) until a constant weight was reached. Measures were done in triplicate in the same zones of cheeses (rind, underrind and inner part of the cheese) sampled for the texture analysis.

7.3.4 Data processing

7.3.4.1 Multivariate data processing

Principal Component Analysis (**PCA**) was applied as an exploratory analysis to observe a possible classification among samples with different rind percentages. To build up chemometric models of rind and particle size parameters' quantification, PLS regression technique (SIMPLS algorithm) was chosen and performed on NIR absorbance spectra and IA parameters of grated cheeses. PLS regression is a multivariate technique used to build predictive models when independent variables (spectral data, X-array) are many and collinear (Menesatti et al., 2010). This regression technique applies variables decomposition to reduce the quantity of given information by spectral data and image analysis parameters, relating X-array to a Y-vector (response variable). The result is the creation of a number of Latent Variables (**LVs**) that are a reconstruction of the original spectral data and that are highly correlated with the Y-response. Different pre-processing transformations of absorbance spectra and IA parameters were evaluated (mean and median centering, auto-scaling, baseline correction, detrending, multiplicative scattering correction, extended multiplicative scattering correction, standard normal variate, Savitzky–Golay 1^{st}-2^{nd} derivative, generalized least squares weighting). Cross-validation techniques (random sub-set, leave-one-out) were used to assess

the best pre-processing method and to select the optimal number of LVs, by considering the Root Mean Square Error in Cross Validation (**RMSECV**) and the coefficient of determination (R^2). In addition, the root-mean-square error of calibration (**RMSEC**) was included in the calibration statistics to assess model goodness (Niu et al., 2008). For IA PLS model, Variable Importance in the Projection (**VIP**) scores that express the importance of each variable in the definition of model's LVs (Gerretzen et al., 2016) were calculated according to equation 1:

$$VIP_k = \sqrt{K \sum_{f=1}^{f} \frac{w_{kf}^2 \cdot SSY_f}{SSY_t}} \quad (1)$$

Where K is the total number of X parameters, w_{jf} is the weight value for k^{th} parameter and f^{th} latent variable, SSY_f is the sum of squares of explained variance by k^{th} parameter for f^{th} component and SSY_t is the total sum of squares explained of the dependent variable.

The developed calibration model based on rind quantification was finally validated to predict the percentage of rind of the samples in an independent testing set (prediction set). To perform validation, samples were divided prior to calibration into a calibration set (66%) and a prediction set (34%) based on Kennard–Stone sampling algorithm (Kennard & Stone, 1969). Because of the lower number of samples, models based on particle size parameters were not externally validated. All multivariate analyses were performed using PLS Toolbox v. 8.0 (Eigenvector Research Inc., WA, USA) with MATLAB V8.5.0 R15a (The Math Works, Natick, USA).

7.3.4.2 Statistical analyses

Two-way Analysis of Variance (ANOVA) was performed to assess statistical differences based on textural, particle size and moisture data as function of different ages and zones in cheese geometry or percentages of rind. Univariate statistical analyses were carried out using PRC GLM of SAS (SAS Inst. Inc., NC, USA); lsmeans with LSD adjustment was used to perform multiple comparisons among means. Pearson correlation coefficients were calculated among the measured parameters using SPSS v.25.0 (SPSS Inc., Armonk, USA). Before statistical procedures, data to be analyzed were tested for normality and homogeneity of variance.

7.4 Results and Discussion

7.4.1 Relation among textural properties, moisture content and particle size

Samples showed clear differences in textural properties among different zones of the cheese. As expected, rind showed greater hardness at rupture (H_r), maximum hardness (H_{max}), positive work area (A_p), initial slope (S_{ini}) and modulus (Mod_1) than underrind and inner part of the cheese (**Table 2**); underrind was slightly harder than inner part of the cheese although H_r, H_{max} values were not significantly different for almost all the comparisons. The inverse relation between moisture content and hardness was already pointed out for parmesan-type cheeses (Jaster et al., 2014) but was not yet investigated for different zones of the cheese wheel. A higher firmness of cheese rind than the inner part of the cheese is related to the highly different moisture content of the zones of the cheese; in facts, the mean difference of moisture content between the inner and the rind part of the cheese was 14.01±1.98%. Differences of moisture between the inner and the outer zones of the wheel may also affect bacterial growth and biochemical reactions leading to different sensory properties among different cheese zones (Cattaneo et al., 2008; Lindner et al., 2008; Malacarne et al., 2009, Mayer, 1996). Consequently, texture may affect the result of grating operations in terms of dimension and shapes of the particles. The rind showed higher values of distance at rupture (D_r) indicating that this zone was more elastic than the inner, which was found to be more brittle (Patel, Upadhyay, Miyani, & Pandya, 1993). Ripening time affects both textural and moisture content results and consequently H_r and moisture were strongly inversely related (**Table 3**). Despite a stronger texture and a lower moisture content is expected as the ripening time increases, this relation between time and cheese properties can be always true only for the same batch of cheese. In facts, in particular for outer cheese's zones (rind and underrind), a high variability in textural properties and moisture content was observed among different ripening times and no clear relations were highlighted, likely due to the high variability in P-R cheeses that can be partially caused by the use of milk without standardized chemical composition and by dairy specificity practices according to P-R cheese-making technology (Mucchetti et al., 2014); for instance, samples at 18 and 24 months of ripening were found to be firmer and drier than samples at 12 and 21 months, respectively. Despite of that, samples at 30 months were nearly two-times harder and drier than samples at 12 months. Textural properties affected size and dimensions of the particles deriving from the grating procedure. Grated samples with different ripening time and percentages of rind (0, 18, 100%) showed differences in IA parameters, as reported in **Table 4**. Number of particles (n) and total surface covered by particles (S_T) present in the same amount of cheese (0.3 g) were highly positively influenced by rind content.

Grated rind fraction showed a largely higher amount of finer, small particles (F and mF <0.5mm) and consequently a lower number of larger particles (F and mF in the range between 0.5-1mm and 1-2mm). Considering different ripening times, no clear trends were highlighted among samples as well as for textural parameters and moisture content; however, significantly strong positive correlations among n, S_T, $F_{<0.5mm}$, $mF_{<0.5mm}$ and H_r, D_r were observed. A negative correlation between the above-mentioned IA parameters and moisture content was also highlighted (**Table 3**). It has been reported in literature that a higher firmness can produce lower particle sizes as the result of grinding/grating operations in the case of wheat milling (Campbell, Fang & Muhamad, 2007; Fang, & Campbell, 2003; Haddad, Mabille, Mermet, Abecassis, & Benet, 1999); in facts, the result of these operations could be affected by product's hardness and moisture. Also, mean circularity (C) was slightly affected by grinded cheese zone although it was not always statistically different; grated rind showed lower circularity values than the inner part of the cheese and this is because rind's particles with F and mF bigger than 0.5 mm tends to be characterized by a more elongated, fibrous shape (**Figure 1**).

7.4.2 PLS Models development for the prediction of rind percentage and particle size properties

7.4.2.1 Rind percentage determination based on particle size measurements

Considering the different particle characteristics observed for rind and the inner part of the cheese (see paragraph above), PLS regression was performed to build up a quantitative model based on IA parameters for the determination of the rind content in grated cheese samples (**Table 5**). Autoscaling was chosen as preprocessing method; model resulted in 2 LVs that explained 87.22% and 87.81% of total variance for X- and Y-block, respectively. Calibration determination coefficient (R^2_{cal}) was 0.88, while Cross-Validation determination coefficient (R^2_{cv}) and RMSECV were 0.86 and 4.17%, respectively. Model's goodness of fit can be considered good (Karoui et al., 2006). VIP scores **(Fig. 2)**. showed that C, S_T, n, $F_{0.5\text{-}1mm}$, $F_{>2mm}$ were the most relevant variables having a score higher than 1, while the other mF and F parameters did not seem to be significant in determining the percentage of rind. Results highlighted a higher error than those reported by other authors with NIRs (Barzaghi et al., 2016; Cevoli, Fabbri, Gori, Caboni, & Guarnieri, 2013; Musi, & Filippi, 2015) but showed the feasibility of IA to be a fast and economic complementary analysis to detect the rind percentage in grated P-R cheese.

7.4.2.2 Preliminary analyses of NIR spectra

The effect of different rind percentages on NIR spectra of grated cheese samples is shown in **Figure 3a**. Averaged raw spectra containing 4 different rind percentages (0, 9, 18, 27, 36%) of the whole dataset showed similar spectral behaviour and were characterized by the main absorption bands of water and fat (Cevoli, Gori, Nocetti, Cuibus, Caboni, & Fabbri, 2013). A decreasing baseline height of the spectrum was highlighted, as the rind content of the sample increases because of the increasing percentage of particles with lower dimensions (Holroyd, 2013; Silaghi, Giunchi, Fabbri, & Ragni, 2009). Moreover, a high difference in absorbance values was observed at water absorption wavelengths (1936 nm and 1457 nm); samples with a higher content of rind showed lower absorbance values because of their lower moisture content. Performing a first derivate on raw spectra (**Figure 3b**), contributions from the lipids, water and proteins (bands located around 1885 nm and 2190 nm attributed to the water absorption in the 1st overtone and N–H and O–H bonds, respectively), as reported by other authors (Downey et al., 2005; Karoui et al., 2006), can be clearly observed. Spectral differences were also investigated by PCA analysis to explore the effect of increasing amount of rind in the samples (**Figure 4a)**. Observing the scores plot graph, a good separation among samples with different rind percentages was highlighted; in particular, samples were greatly discriminated by PC 1, that explained 98.29% of the total variance among the samples.

7.4.2.3 Prediction of Image Analysis parameters based on NIR analysis

PCA scores plot of grated cheese NIR spectra showed a good separation among samples characterized by low and high number of particles (**n**) as shown in **Figure 4b**. Moreover, a good discrimination of treated (further grinded) and untreated grated cheese samples was obtained with PCA scores plot (results not shown) as a consequence of light scattering phenomena (data not shown). Considering the effect of light scattering over NIR spectra, PLS models for the prediction of particle size based on IA measurements were built up (**Table 5**). Models for the prediction of n and S_T parameters gave good results both in calibration and cross-validation, as R^2 were higher than 0.828; moreover, models were considerably robust and stable as were constituted by only 1 latent variable that explained a large amount of response variance. Best preprocessing method of NIR spectra was mean centering and autoscaling of data for all particle size models, respectively. As expected, data pretreatment involving scattering correction (1st derivative, multiple scattering correction, baseline adjustment) gave worst results. Models concerning frequency percentage estimation of F and mF at

different class sizes (<0.5mm, 0.5-1mm, 1-2mm, >2mm) did not give good results, as R^2 was in the range between 0.5-0.7 both in calibration and cross-validation, with the exception of $mF_{>2mm}$ that gave a determination coefficient higher than 0.8; these reported results can be caused by a non-homogeneous distribution of y-values across the calibration range. Additionally, the ratio of prediction to deviation (RPD, not shown) of these models was lower than 2, except for $mF_{>2mm}$. Thus, models for the prediction of frequency percentages classes based on mF and F were able only to discriminate between low and high values (Karoui et al., 2006).

7.4.2.4 NIR analysis for the prediction of rind content

Concerning the prediction of rind with NIRs, data were preprocessed using 1st derivative, multiple scattering correction followed by autoscaling or 1st derivative, standard normal variate followed by autoscaling; the two different preprocessing procedures gave similar results (**Table 5**). Moreover, for each preprocessing method 2 different PLS models were built up: a model that processed the whole acquired NIR spectrum (1000-2500 nm) of the samples and a model based on the selection of a sub-region located in two wavelength intervals (1064 - 1335 nm, 1933 - 2357 nm) to reduce the complexity of PLS models and reach a desired error level (Jiang, Berry, Siesler, & Ozaki, 2002). The selection of informative regions aims to further improve the prediction ability of the PLS models than those of the models constructed on the whole spectral points (Du, Liang, Jiang, Berry, & Ozaki, 2004). The excluded wavelength intervals mostly refer to water absorption IR bands that, in the case of P-R, can be subjected to fluctuations because of moisture content variability, that is influenced by cheesemaking and ripening conditions (**Table 2**). Indeed, the PLS models built up on the selected sub-region were more robust than models including the entire absorption spectrum, as they were constituted by 4 LVs instead of 5. R^2 values were higher than 0.93 in calibration and cross-validation while slightly decreased in validation (0.87). Taking into account the RMSEP values (3.44-3.45%), models' performances can be considered good and were similar to other reported in literature (Barzaghi et al., 2016; Cevoli, Fabbri, Gori, Caboni, & Guarnieri, 2013; Cevoli, Ragni, Gori, Berardinelli, & Caboni, 2012).

7.4.3 Application of NIR Model to predict the percentage of rind during industrial grating operations

A simple calculation of the volume ratio between the rind and the inner part of the whole cheese, considering the thickness of 6 mm of the rind and the variability of the dimension of the wheel that are established by PDO Regulation (diameter of the faces from 35 to 45 cm and heel height from 20 to 26 cm), shows that this ratio is largely less than 18% also for the smallest wheel. Thus, the limit of 18% of rind in grated cheese can be surpassed because of mixing errors before grating when both whole wheels and trimmed parts are used and/or variability caused by different sedimentation velocities of grated cheese particles with different size or densities during packaging operations.

NIRs measurements applied to industrial grating operations showed differences both as a function of cheese batch and process time from the start of grating operations ($P < 0.05$); as reported in **Figure 5**, no clear trends were observed in samples collected at different process times. A high variability was observed among grating operations carried out on different cheese batches, ranging from 9.19 ± 1.77% of batch 1 to 17.36 ± 1.68% of batch 7; this can be attributed to the variability that is obtained during the loading operations of both trimmed pieces of cheese and whole wheels that are addressed to the grater. Moreover, intra-treatment variability was also visible for some samples, as highlighted by samples standard deviation and can be caused by the reported error of PLS model based on NIR spectroscopy. The first six product batches amply fell within the rind limit content of 18%, except for batch 7 which was close to the limit. The NIRs model here developed proved to rapidly provide an indication of rind percentage before the products are released in the market, allowing to further check and manage non-compliant products.

7.5 Conclusions

This study enabled to get a better understanding about the physical properties of grated P-R cheese, highlighting the different characteristics of cheese particles generated by the rind and by the inner part of the cheese; moreover, it was pointed out that these differences were highly related to the different texture and moisture content between the inner and the rind zone of the cheese. PLS models built on selected sub-region of the NIR spectra in order to minimize water absorption bands, proved to successfully predict the rind content but also the particle size characteristics of grated P-R cheese and can be used to monitor the industrial grating process, with both inline and offline applications. Image analysis was useful to study the physical characteristics of the cheese particles and allowed to develop PLS stable models to predict number of particles and total surface covered by the particles. Both NIRs and IA proved to be reliable tools that could be employed by grated P-R cheese producers as internal quality control measurements to efficiently comply with both PDO regulation and quality programs.

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7.8 Figures and tables

Figure 1. Details of image acquired for rind (**a**) and inner part (**b**) of grated P-R cheese after 24 months of ripening.

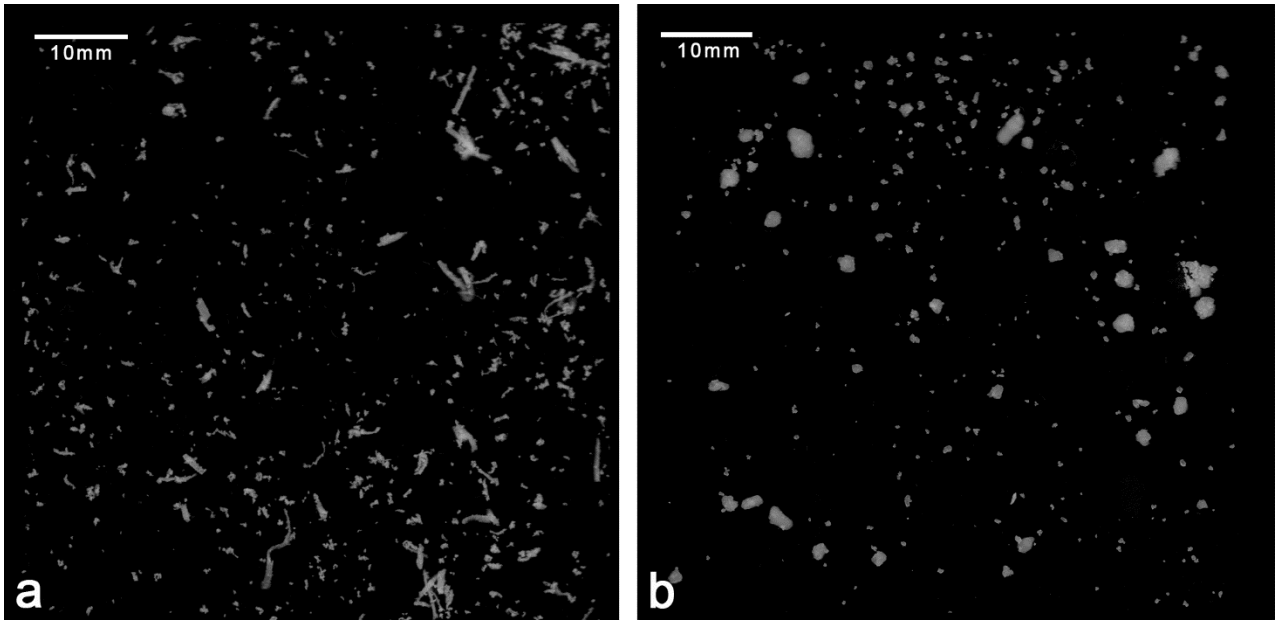


Figure 2. VIP (Variable Importance in the Projection) scores of Image Analysis variables (total number of particles **n**, total surface covered by particles S_T, circularity **C**, Feret diameter **F** and minimum Feret diameter **mF**) for the model built for the quantification of rind percentage in Parmigiano Reggiano samples. The VIP scores shows the relative importance of each variable in the projection of model's LVs.

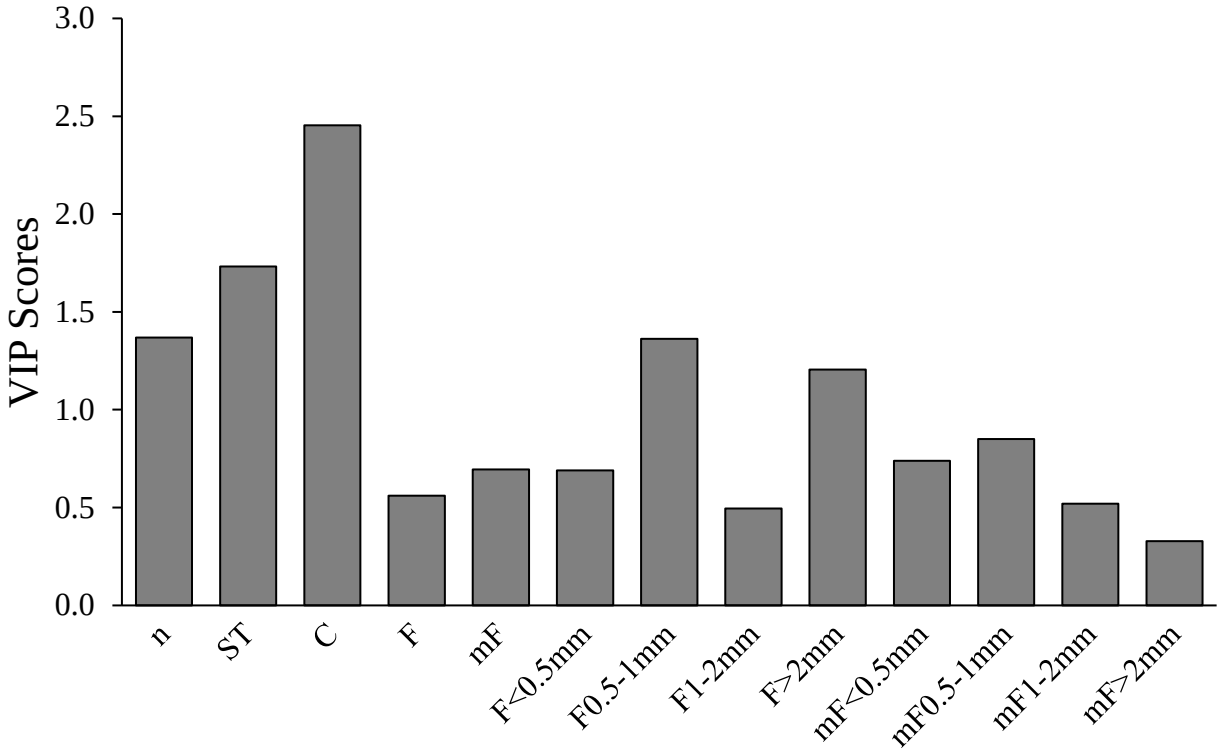


Figure 3. Averaged raw spectra (**a**) and Savitzky–Golay first derivative (**b**) of grated cheese having different rind percentages (w/w), namely 0% (_____), 9% (.....), 18% (_ _ _ _), 27% (_ . . _), 36% (_ _ _).

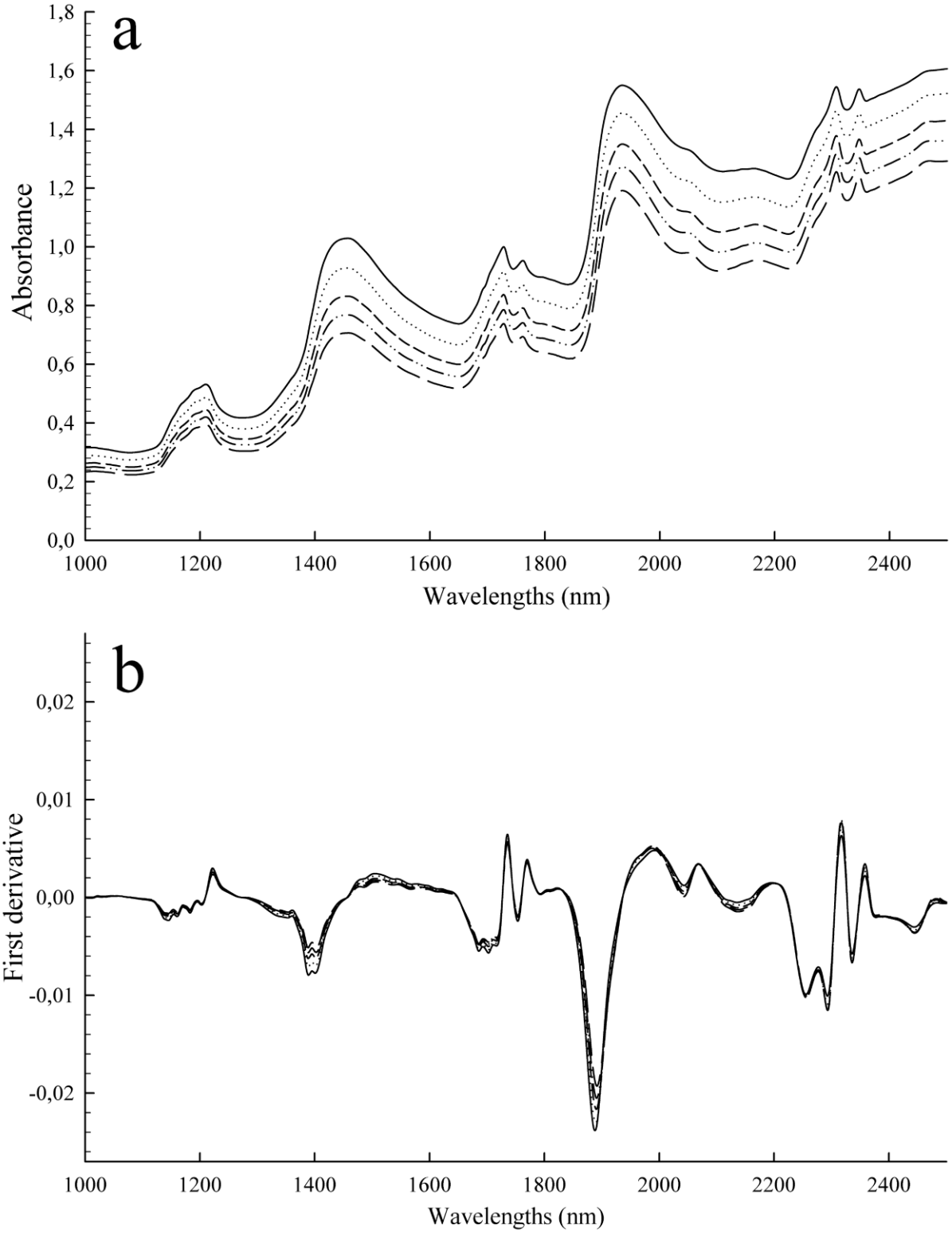


Figure 4. Principal Component Analysis (PCA) scores plot of grated cheese samples having different rind percentages (w/w), namely 0%, 9%, 18%, 27% and 36% (**a**), and characterized by a different number of particles in 0.3g of sample (**b**).

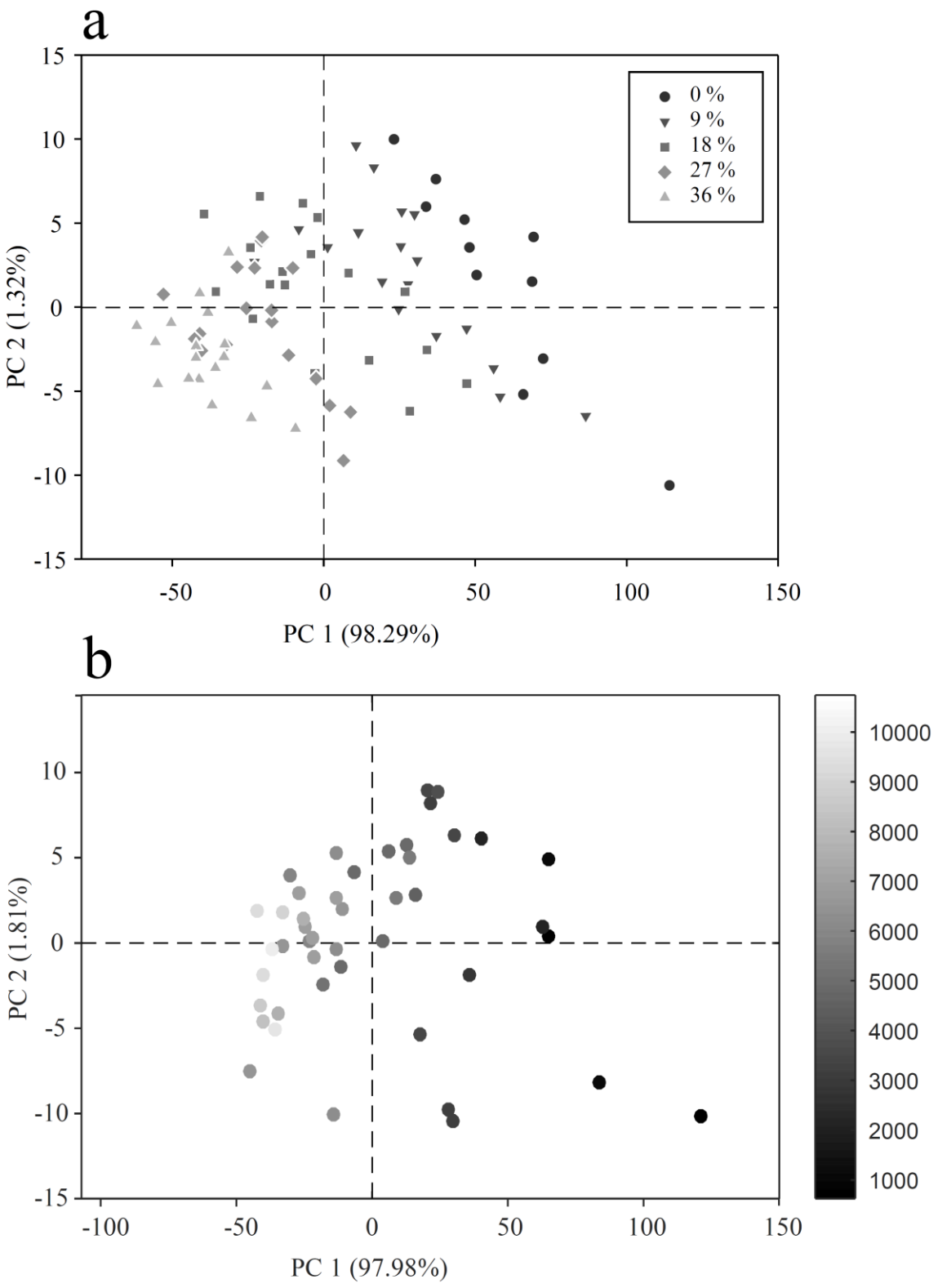


Figure 5. Percentage of rind of grated P-R cheese samples produced from 7 different grating processes and measured at 5 different process times, namely t0, t1, t2, t3, t4, t5.

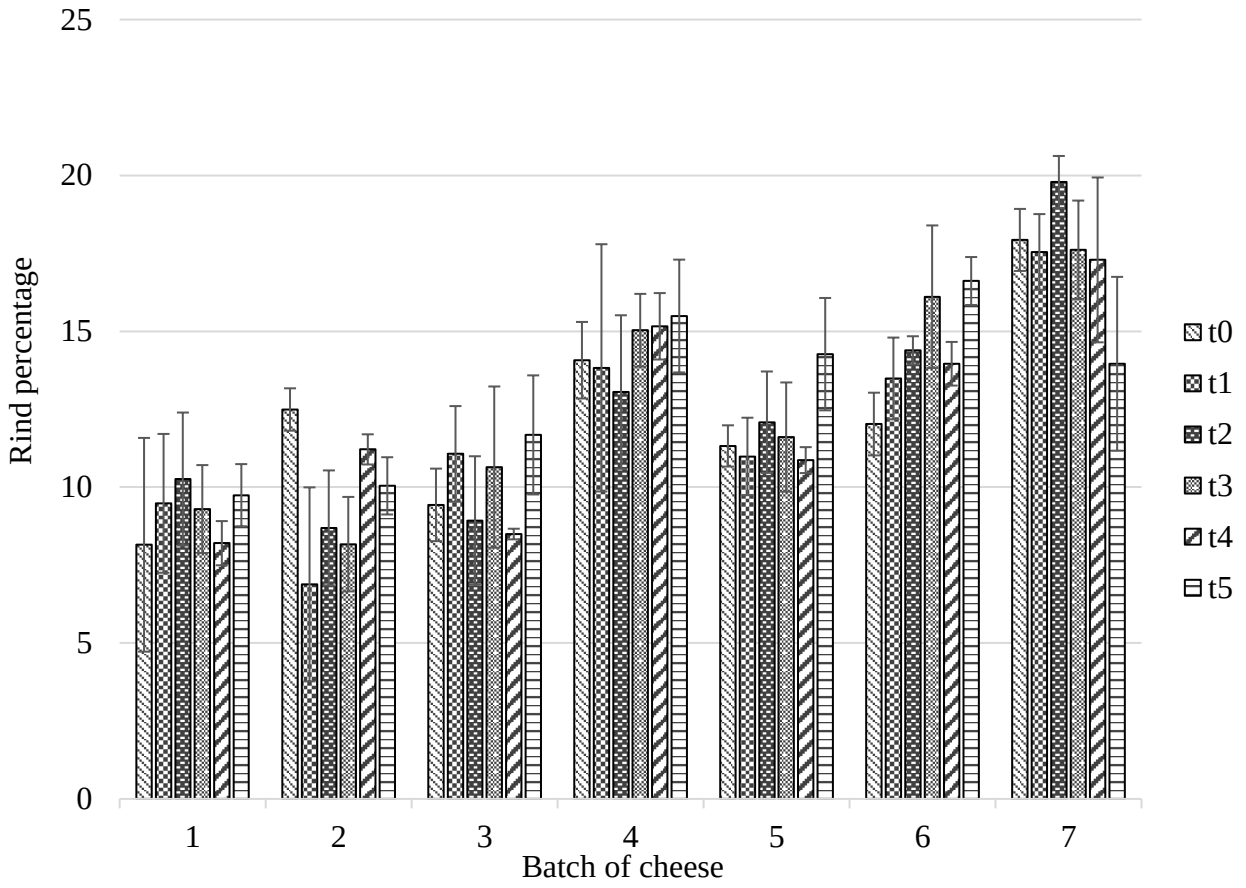


Table 1. Number of grated Parmigiano-Reggiano cheese samples analysed by Near-Infrared spectroscopy and image analysis reported for every ripening time and rind percentage level considered in this study.

Percentage of rind of the sample (w/w)	Ripening time of sampled rind and inner part (months)	Number of samples analysed by NIR spectroscopy	Number of samples analysed by Image Analysis
0	12, 18, 21, 24, 30	11	10
4	12, 24, 30	5	–
5	13,21,24	4	–
9	12, 18, 21, 24, 30	18	10
10	12, 24, 30	9	–
15	13,21,24	4	–
16	12, 24, 30	5	–
18	12, 18, 21, 24, 30	18	10
20	12, 24, 30	9	–
25	13,21,24	4	–
27	12, 18, 21, 24, 30	18	10
30	12, 24, 30	8	–
36	12, 18, 21, 24, 30	18	10

Table 2. Textural parameters (hardness at rupture (H_r), distance at rupture (D_r), maximum hardness (H_{max}), distance at maximum force (D_{max}), initial slope (S_{ini}), modulus between the origin and the maximum force (Mod_1) and positive work area (A_p)) and moisture content of Parmigiano Reggiano cheese samples having different ripening times; results (mean ± standard deviation) are expressed for different zones of the cheese.

Ripening time (months)	Wheel zone	H_r (N)	D_r (mm)	H_{max} (N)	D_{max} (mm)	S_{ini} (N mm^{-1})	Mod_1 (-)	A_p (N mm)	Moisture (% w/w)
	inner	4.03 e ± 0.37	0.88 c ± 0.11	5.60 ef ± 0.36	9.94 a ± 0.09	6.31 e ± 1.36	11.41 f ± 0.12	44.20 f ± 3.05	32.00 a ± 1.38
12	underrind	6.78 de ± 1.18	1.26 c ± 0.15	9.65 def ± 1.39	10.00 a ± <0.01	10.03 de ± 2.63	13.92 d ± 1.01	76.39 ef ± 12.28	26.67 cd ± 2.32
	rind	36.68 c ± 6.55	3.51 a ± 0.42	36.68 c ± 6.55	3.51 c ± 0.42	22.45 c ± 4.83	36.85 a ± 6.52	295.45 cd ± 59.90	18.05 fg ± 2.16
	inner	3.50 e ± 0.39	1.19 c ± 0.64	5.26 f ± 0.63	9.99 a ± 0.02	5.54 e ± 1.71	11.30 f ± 0.30	40.45 f ± 4.52	32.13 a ± 0.33
18	underrind	5.37 de ± 1.32	1.23 c ± 0.65	8.32 ef ± 1.67	9.75 ab ± 0.6	7.89 de ± 2.97	12.88 de ± 1.13	63.76 ef ± 12.41	28.19 bc ± 0.93
	rind	35.45 c ± 8.10	3.58 a ± 0.46	35.46 c ± 8.10	3.63 c ± 0.45	21.94 c ± 5.12	35.66 a ± 8.04	267.60 d ± 70.12	20.57 ef ± 1.98
	inner	4.42 de ± 0.45	1.20 c ± 0.62	6.67 ef ± 0.58	9.24 ab ± 1.84	5.90 e ± 3.45	11.45 e ± 1.46	51.50 f ± 3.25	31.46 ab ± 0.80
21	underrind	7.23 de ± 1.19	1.14 c ± 0.37	11.42 de ± 1.93	9.46 ab ± 1.29	12.50 de ± 3.21	14.88 c ± 1.81	89.45 e ± 15.43	26.10 cd ± 2.22
	rind	49.88 b ± 8.67	3.75 a ± 0.94	49.88 b ± 8.67	3.75 c ± 0.94	30.24 b ± 10.86	50.04 a ± 8.61	391.56 b ± 70.34	15.27 gh ± 1.02
	inner	4.53 de ± 0.71	1.03 c ± 0.20	6.68 ef ± 0.37	9.37 ab ± 1.50	6.98 e ± 2.76	11.57 e ± 0.94	52.34 ef ± 2.30	29.02 abc ± 1.64
24	underrind	6.33 de ± 0.73	1.13 c ± 0.6	10.86 de ± 1.43	8.91 b ± 2.14	9.75 de ± 3.74	14.20 c ± 1.19	84.56 ef ± 11.88	27.07 c ± 3.48
	rind	45.66 b ± 6.79	3.47 a ± 0.4	45.66 b ± 6.79	3.47 c ± 0.40	26.99 b ± 3.42	45.79 a ± 6.78	337.58 bc ± 62.09	16.42 gh ± 2.07
	inner	5.58 de ± 0.73	1.27 c ± 0.51	8.87 ef ± 2.10	9.76 ab ± 0.58	7.43 de ± 2.98	13.30 d ± 1.17	66.24 ef ± 10.18	29.59 abc ± 1.90
30	underrind	10.68 d ± 2.59	2.19 b ± 0.88	15.25 d ± 3.18	9.99 a ± 0.01	10.39 d ± 3.19	18.29 b ± 2.76	117.44 ef ± 25.10	23.49 de ± 2.17
	rind	61.02 a ± 11.62	3.98 a ± 0.60	61.02 a ± 11.62	3.98 c ± 0.60	38.42 a ± 9.30	61.16 a ± 11.57	483.37 a ± 97.82	13.85 h ± 1.13

Multiple comparisons were performed using GLM procedure with lsmeans option with LSD adjustment (SAS Institute, Cary, North Carolina). Means with different superscripts for each textural parameter considered (column) are statistically different ($P < 0.05$).

Table 3. Pearson correlation coefficients for particle size and textural parameters evaluated for rind and inner part of Parmigiano Reggiano cheese samples. Distance at rupture (D_r), moisture content, total number of particles (n), total surface covered by particles (S_T), circularity (C), Feret diameter (F) and minimum Feret diameter (mF) are displayed.

	D_r (mm)	Moisture % (w/w)	n (-)	S_T (mm^2)	C (-)	$F_{<0.5mm}$ (%)	$F_{0.5-1mm}$ (%)	F_{1-2mm} (%)	$F_{>2mm}$ (%)	$mF_{<0.5mm}$ (%)	$mF_{0.5-1mm}$ (%)	mF_{1-2mm} (%)	$mF_{>2mm}$ (%)
H_r (N)	0.820**	-0.961**	0.934**	0.913**	-0.726**	0.794**	-0.869**	-0.652**	-0.083	0.765**	-0.811**	-0.592**	-0.460*
D_r (mm)		-0.886**	0.897**	0.909**	-0.711**	0.807**	-0.891**	-0.668**	-0.038	0.780**	-0.828**	-0.603**	-0.455*
Moisture % (w/w)			-0.966**	-0.955**	0.774**	-0.849**	0.913**	0.727**	0.181	-0.828**	0.865**	0.679**	0.576*
n (-)				0.994**	-0.765**	0.900**	-0.941**	-0.778**	-0.183	0.884**	-0.922**	-0.719**	-0.581**
S_T (mm^2)					-0.796**	0.887**	-0.929**	0-.766**	-0.178	0.879**	-0.915**	-0.717**	-0.589**
C (-)						-0.485**	0.609**	0.338	-0.135	0.518*	0.563**	0.356	0.312
$F_{<0.5mm}$ (%)							-0.935**	0-.956**	-0.449*	0.987**	-0.991**	-0.895**	-0.736**
$F_{0.5-1mm}$ (%)								0.798**	0.121	-0.888**	0.942**	0.692**	0.496*
F_{1-2mm} (%)									0.630**	-0.974**	0.944**	0.966**	0.827*
$F_{>2mm}$ (%)										-0.520*	0.392*	0.753**	0.860**
$mF_{<0.5mm}$ (%)											-0.988**	-0.942**	-0.801**
$mF_{0.5-1mm}$ (%)												0.881**	0.709**
mF_{1-2mm} (%)													0.920**

* P<0.05

** P<0.001

Table 4. Image Analysis parameters of Parmigiano Reggiano grated cheese samples having different ripening times; results (mean ± standard deviation) of total number of particles (**n**), total surface covered by particles (S_T), circularity (**C**), Feret diameter (**F**) and minimum Feret diameter (**mF**) are expressed as a function of the different rind percentage present in the samples.

Ripening time (months)	Rind % (w/w)	n (-)	S_T (mm^2)	C (-)	$F_{<0.5mm}$ (%)	$F_{0.5-1mm}$ (%)	F_{1-2mm} (%)	$F_{>2mm}$ (%)	$mF_{<0.5mm}$ (%)	$mF_{0.5-1mm}$ (%)	mF_{1-2mm} (%)	$mF_{>2mm}$ (%)
12	0	1899i ±78	631h ±17	0.84bc ±0.01	42.75i ±1.59	38.76a ±1.77	15.54b ±0.87	2.95c ±0.14	66.19g ±1.3	27.50b ±1.11	5.38b ±0.58	0.93b ±0.29
	18	4714g ±292	1033f ±10	0.83cde ±0.01	53.72efg ±1.79	32.27ef ±1.19	11.48de ±0.55	2.53cde ±0.33	77.96de ±1.08	18.67cd ±0.90	2.99de ±0.35	0.38cd ±0.08
	100	18028c ±881	2701c ±217	0.83cde ±0.01	65.47a ±1.40	24.26g ±0.80	8.05h ±0.59	2.21def ±0.30	87.50a ±0.78	10.79f ±0.49	1.55g ±0.27	0.16de ±0.07
18	0	881i ±174	432i ±33	0.85b ±0.01	39.53i ±2.70	36.18bc ±0.78	18.67a ±2.06	5.62a ±0.88	59.30h ±3.09	29.81a ±1.80	8.90a ±1.33	1.99a ±0.45
	18	3306h ±107	834g ±14	0.82ef ±<0.01	51.06g ±2.33	32.32ef ±2.08	13.08cd ±0.4	3.55b ±0.39	77.08e ±0.93	19.22cd ±1.14	3.17d ±0.22	0.53c ±0.08
	100	17317c ±187	2755c ±35	0.82f ±<0.01	61.25bc ±0.22	26.55g ±0.53	9.46gh ±0.31	2.75cd ±0.03	85.42ab ±0.09	12.47f ±0.05	1.93fg ±0.07	0.17de ±0.01
21	0	3911gh ±75	785gh ±57	0.87a ±0.01	56.15def ±2.62	33.13de ±1.61	9.56gh ±0.91	1.16ij ±0.21	79.34de ±1.91	18.15d ±1.47	2.22efg ±0.40	0.29cde ±0.06
	18	6865e ±574	1203e ±143	0.85b ±0.01	58.68cd ±1.50	30.20f ±0.55	9.53gh ±1.34	1.59ghi ±0.25	83.02bc ±1.89	15.16e ±1.68	1.65g ±0.23	0.18de ±0.03
	100	19378b ±881	2916b ±143	0.82f ±0.01	61.66bc ±3.50	25.83g ±1.93	9.68fgh ±1.21	2.83c ±0.37	85.44ab ±1.97	12.34f ±1.76	2.07fg ±0.17	0.14de ±0.04
24	0	3246h ±320	834g ±5	0.85b ±0.01	46.57h ±4.02	37.70ab ±2.22	14.12bc ±1.66	1.61ghi ±0.22	69.46f ±3.11	25.89b ±2.12	4.42c ±0.99	0.23de ±0.04
	18	6418ef ±101	1243e ±61	0.84bcd ±0.01	52.87fg ±1.36	34.90cd ±0.95	10.76efg ±0.89	1.47hi ±0.08	78.74de ±1.58	18.94cd ±1.38	2.23efg ±0.21	0.09e ±<0.01
	100	19162b ±953	2831bc ±135	0.83ef ±<0.01	62.93ab ±0.89	26.10g ±0.55	8.94h ±0.43	2.03efg ±0.04	85.92a ±0.52	12.37f ±0.42	1.60g ±0.08	0.12e ±0.04
30	0	5805f ±479	1129ef ±76	0.85b ±<0.01	51.92g ±2.97	35.92bc ±2.83	11.25ef ±0.46	0.91j ±0.31	76.79e ±1.69	20.57c ±1.88	2.53def ±0.20	0.11e ±0.05
	18	9070d ±717	1585d ±66	0.83cde ±<0.01	56.83de ±0.69	31.17ef ±0.57	10.62efg ±0.17	1.38hij ±0.17	80.47cd ±0.76	17.24de ±0.65	2.20efg ±0.06	0.10e ±0.06
	100	22447a ±1473	3184a ±107	0.83def ±0.01	64.94ab ±2.41	24.70g ±1.26	8.47h ±0.86	1.89fgh ±0.30	86.94a ±1.33	11.28f ±1.03	1.66g ±0.25	0.12e ±0.06

Multiple comparisons were performed using GLM procedure with lsmeans option with LSD adjustment (SAS Institute, Cary, North Carolina). Means with different superscripts for each parameter considered (column) are statistically different ($P < 0.05$).

Table 5. Partial least squares regression results using cheese NIR spectra and Image Analysis parameters to predict rind percentage and particle size properties of Parmigiano Reggiano grated cheese samples.

Data source	Model	Spectral range	Preprocessing method	Calibration data set			Calibration				Cross-validation			Validation		
				Range	Mean	SD	n	LVs	R^2	SEC	R^2	RMSECV	n	R^2	RMSEP
Image Analysis	Rind percentage (% w/w)	-	Autoscale	-	-	-	50	2	0.880	3.90	0.860	4.17	-	-	-
NIR	Rind percentage (% w/w)	1000 - 2500 nm	1st Derivative (Sav-Gol) + Multiplicative Scattering Correction + Autoscale	-	-	-	87	5	0.956	2.72	0.914	3.24	44	0.847	3.82
NIR	Rind percentage (% w/w)	1064 - 1335 nm, 1933 - 2357 nm	1st Derivative (Sav-Gol) + Multiplicative Scattering Correction + Autoscale	-	-	-	87	4	0.952	2.42	0.936	2.86	44	0.875	3.44
NIR	Rind percentage (% w/w)	1000 - 2500 nm	1st Derivative (Sav-Gol) + Standard Normal Variate + Autoscale	-	-	-	87	5	0.952	2.38	0.925	3.00	44	0.858	3.69
NIR	Rind percentage (% w/w)	1064 - 1335 nm, 1933 - 2357 nm	1st Derivative (Sav-Gol) + Standard Normal Variate + Autoscale	-	-	-	87	4	0.952	2.41	0.934	2.89	44	0.874	3.45
NIR	n (-)	1000 - 2500 nm	Mean Center	620 - 10743	5507	2510	46	1	0.851	969	0.828	1045	-	-	-
NIR	S_T (mm^2)	1000 - 2500 nm	Autoscale	334 - 1731	1087	342	46	1	0.872	122	0.855	130	-	-	-
NIR	$F_{<0.5mm}$ (%)	1000 - 2500 nm	Mean Center	34.93 - 64.76	52.55	7.16	46	1	0.607	4.49	0.497	5.15	-	-	-
NIR	$F_{0.5-1mm}$ (%)	1000 - 2500 nm	Mean Center	26.70 - 43.71	33.53	4.14	46	2	0.602	2.61	0.504	2.94	-	-	-
NIR	F_{1-2mm} (%)	1000 - 2500 nm	Mean Center	7.53 - 20.05	11.64	2.96	46	3	0.615	1.84	0.510	2.10	-	-	-
NIR	$F_{>2mm}$ (%)	1000 - 2500 nm	Mean Center	0.99 - 6.56	2.27	1.27	46	2	0.646	0.76	0.013	0.84	-	-	-
NIR	$mF_{<0.5mm}$ (%)	1000 - 2500 nm	Mean Center	57.30 - 86.66	76.96	7.45	46	1	0.704	4.05	0.633	4.54	-	-	-
NIR	$mF_{0.5-1mm}$ (%)	1000 - 2500 nm	Mean Center	12.01 - 34.50	19.57	5.29	46	2	0.682	2.98	0.583	3.44	-	-	-
NIR	mF_{1-2mm} (%)	1000 - 2500 nm	Mean Center	1.21 - 9.95	3.06	2.00	46	3	0.839	0.80	0.749	0.75	-	-	-
NIR	$mF_{>2mm}$ (%)	1000 - 2500 nm	Mean Center	0.09 - 2.38	0.41	0.52	46	3	0.872	0.18	0.810	0.23	-	-	-

8. Short Educational CV

2004-2009: Scientific high school diploma (84/100), Lyceum Giacomo Ulivi, Viale Maria Luigia 3, 43125 Parma (Italy)

2009-2013: Bachelor's degree in Food Science and Technology (106/110), Department of Agriculture, Università di Parma, Italy.

2013-2016: Master's degree in Food Science and Technology (110/110 summa cum laude), Food and Drug Department, Università di Parma, Italy.

2016-2019: Ph.D. in Food Science, Food and Drug Department, Università di Parma, Italy.

Feb 2019- Jun 2019: Visiting Ph.D. period in University of Aarhus, Department of Food Sciences Denmark and in Arla Innovation Center, Aarhus, Denmark.

8.1Lists of scientific publications

Alinovi, M., Cordioli, M., Francolino, S., Locci, F., Ghiglietti, R., Monti, L., … Giraffa, G. (2018). Effect of fermentation-produced camel chymosin on quality of Crescenza cheese. *International Dairy Journal, 84*, 72–78. https://doi.org/10.1016/j.idairyj.2018.04.001

Alinovi, M., Rinaldi, M., & Mucchetti, G. (2018). Spatiotemporal Characterization of Texture of Crescenza Cheese, a Soft Fresh Italian Cheese. *Journal of Food Quality, 2018*, 1–8. https://doi.org/10.1155/2018/5062124

Rinaldi, M., Cordioli, M., Alinovi, M., Malavasi, M., Barbanti, D., & Mucchetti, G. (2018). Development and Validation of CFD Models of Thermal Treatment on Milk Whey Proteins Dispersion In Batch and Continuous Process Condition. *International Journal of Food Engineering, 14*(9–10). https://doi.org/10.1515/ijfe-2018-0142

Alinovi, M.*, Mucchetti, G., & Tidona, F. (2019). Application of NIR spectroscopy and image analysis for the characterisation of grated Parmigiano-Reggiano cheese. *International Dairy Journal, 92*, 50–58. https://doi.org/10.1016/j.idairyj.2019.01.010

* Publication presented as corresponding author

8.2 Lists of papers under review

Alinovi, M.*, & Mucchetti, G. (2019). A coupled photogrammetric - finite element method approach to model irregular shape product freezing: Mozzarella cheese case. *Journal of Food Engineering.*

Alinovi, M.*, & Mucchetti, G. (2019). Effect of freezing and thawing processes on high-moisture Mozzarella cheese rheological and physical properties. *LWT – Food Science and Technology.*

Alinovi, M., Mucchetti, G., Wiking, L., & Corredig, M. (2019). Freezing of milk, curds and cheese – a review. *Comprehensive Reviews in Food Science and Food Safety.*

Bancalari, E., Alinovi, M., Bottari, B., Caligiani, A., Mucchetti, G., & Gatti, M. (2019). *Frontiers in Microbiology.*

* Publication presented as corresponding author

8.3 Conferences contribution

8.3.1 Oral presentations

Alinovi, M., & Mucchetti, G., (2018). La surgelazione di formaggi freschi a pasta filata per favorire la diffusione del Made in Italy nei mercati lontani. *Milk.it, Quarto convegno nazionale sul mondo del latte*. Cremona, Italy, October 25th, 2018.

Alinovi, M. (2019). Chemometric and computational approaches to improve quality and increase market share of fresh and long-ripened Italian cheeses. *XXIV* Workshop on the *Developments in the Italian PhD Research on Food Science, Technology and Biotechnology*. Firenze, Italy, September 11th-13th, 2019.

8.3.2 Poster presentations

Giraffa, G., Alinovi, M., Carminati, D., Cordioli, M., Francolino S., Ghiglietti, R., Locci, F., Monti, L., Tidona, F., & Mucchetti, G. (2016). Effetti di colture starter e coagulanti innovativi sul processo produttivo della crescenza: resa casearia e caratteristiche. *"Latte e derivati: ricerca, innovazione e valorizzazione" 5th National AITeL conference*. Bari, Italy, 09/09/2016

Alinovi, M. (2017). Mathematical Modeling of Freezing Process in Simple and Complex Food Matrices. *XXII Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology*. Bozen, Italy, September 20th-22nd, 2017.

Alinovi, M. (2018). Properties of Mozzarella Cheese Frozen Under Different Conditions. *XXIII Workshop on the Developments in the Italian PhD Research on Food Science, Technology and Biotechnology*. Oristano, Italy, September 19th-21st, 2018.

Alinovi, M., Mucchetti, G., Andersen, U, Rovers, T. A. M., Mikkelsen, B., Wiking, L., & Corredig, M. (2019). Microstructure changes of processed cream cheeses observed using confocal Raman spectroscopy. *11th Nizo Dairy Conference Milk Protein Functionality*. Papendal, The Netherlands, October 8th-11st, 2019.

8.4Didactic activities

8.4.1 Teaching assistant

Food Technologies 1, part I: Unit Operations. Food Science and Technology Bachelor's Degree course. Food and Drug Department, Università di Parma, Parma, Italy.

Structural and Physical Properties of Foods, part II: Structural and Physical Properties of Foods. Food Science and Technology Master's Degree course. Food and Drug Department, Università di Parma, Parma, Italy.

Designing Methodologies of Food Products and Processing, part I: Designing Methodologies of Food Processing. Food Science and Technology Master's Degree course. Food and Drug Department, Università di Parma, Parma, Italy.

8.4.2 Correlator of Master's thesis in Food Science and Technology

1 Giusti, Giuseppe. Texture and water holding capacity of whey protein gels resulting from different heat treatments. M. D. in Food Science and Technology. Academic Year 2015-2016
2 Zeni, Gabriele. Texture and water holding capacity of whey protein gels obtained in a tubular heat exchanger under different shear rate conditions. M. D. in Food Science and Technology. Academic Year 2015-2016
3 Crescini, Isabella. Physical characterization and evaluation of the stability of emulsioned gels of whey protein. M. D. in Food Science and Technology. Academic Year 2016-2017

4 Giaroli, Chiara. A study of possible connections among milk characteristics, cheese making and presence of Parmigiano Reggiano's crust defects. M. D. in Food Science and Technology. Academic Year 2016-2017
5 Donatini, Marco. NIR spectroscopy applications for characterization of grated Parmigiano Reggiano cheese. M. D. in Food Science and Technology. Academic Year 2016-2017
6 Barosi, Davide. Characterization of grated Parmigiano Reggiano cheese with image analysis and sieving: connections with cheese characteristics. M. D. in Food Science and Technology. Academic Year 2016-2017
7 Bettera, Luca. Comparison between chemical physical and sensory properties of Nostrano Valtrompia PDO cheese aged in different conditions. M. D. in Food Science and Technology. Academic Year 2017-2018
8 Di Nucci, Francesco. Changes in moisture content, water holding capacity and colour of Mozzarella cheese due to freezing and thawing processes. M. D. in Food Science and Technology. Academic Year 2017-2018
9 Grossi, Elena. Freezing and thawing of Mozzarella cheese: characterization of physical and sensory properties. M. D. in Food Science and Technology. Academic Year 2017-2018
10 Sciunzi, Caterina. M. D. in Food Science and Technology. Academic Year 2018-2019
11 Daturi, Simone. M. D. in Food Science and Technology. Academic Year 2018-2019
12 Jeantet, Arianna. M. D. in Food Science and Technology. Academic Year 2018-2019

8.4.3 Didactic laboratories

Rheology and texture laboratory (8 hours). Structural and Physical Properties of Foods (Academic Year 2016-2017)

Rheology and texture laboratory (8 hours). Structural and Physical Properties of Foods (Academic Year 2017-2018)

8.4.4 Tutoring

Student's tutor, according to Regulation 341/90, for the Bachelor's degree in Food Science and Technology. Academic Year 2016-2017.

8.5Learning activities

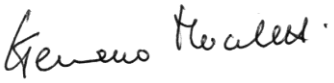
8.5.1 Seminars and workshops

1) Advanced Course in Food Science, Università di Parma, February 2016.
2) Advanced Course in Food Science (summer school), Università di Parma, July 2016.
3) Joint seminar Wageningen-Parma, Università di Parma, October, 18th 2016.

4) Course on Statistics for Food and Nutrition Sciences. Prof. Luigi Palla, from the Imperial College London. Parma, February-March 2017.
5) Advanced Course in Food Sciences. Università di Parma, February 14-16th 2017.
6) . Joint meeting Wageningen University – University of Parma. March, 15th 2017.
7) Introduction to scientific communication. Prof. Bettini e Prof. Lodola. Università di Parma, 29th September 2017.
8) Joint seminar Parma-Wageningen, 18th October 2018. Centre S. Elisabetta, Pad. 13. Via Parco Area delle Scienze 93/A, Parma, Italy.
9) MATLAB Academic tour, 29th October 2018. Room F, Department of Engineering, Parco Area delle Scienze, 69/a, Parma, Italy.

Marcello Alinovi, PhD student

Prof. Germano Mucchetti, supervisor



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Marcello Alinovi, October, 2019