University of Naples
FEDERICO II

Department of Agricultural Sciences

PhD thesis

Agricultural and Food Sciences

(29th cycle)

On the possibility to trace frozen curd in Buffalo Mozzarella PDO cheese

Tutor: prof. Romano Raffaele

Candidate: Manzo Nadia

Coordinator: prof. D'Urso Guido

2014-2017

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1. Introduction

1.1 Problem definition

Campana Buffalo Mozzarella PDO (Protected Designation of Origin) is a stretched curd cheese produced exclusively by using fresh buffalo milk, which must be processed within 60 hours after milking (EC Regulation 103/2008). The PDO trademark was conferred in 1996 due to its typical organoleptic properties.
According to this specification, the cheese must satisfy certain criteria, such as white porcelain color, fat/dry matter ≥ 52% (w/w) and maximum moisture content 65% (w/w).
Geographical Indications are the authentic expression of the values and the history of a territory. These certifications represent a guarantee of quality for both producers and consumers, in terms of safety, genuineness and freshness.

Nowadays many national consortia have to deal with market saturation problems. On the contrary, Campana Buffalo Mozzarella Consortium is worried for the limits to its expansion. Mozzarella cheese is in fact a widespread product: in the year 2015, more than 41,000 tons of cheese were produced (data obtained from the Consortium for the protection of Buffalo Mozzarella PDO cheese).
However, the high consumer's demand of this cheese, the limited amount of available buffalo's milk, and the high market price, make mozzarella cheese a remunerative target for either adulterated or false PDO sales (Mazzei and Piccolo 2012).
It is known that buffalo milk production changes with a seasonal trend. During the summer there is a limited availability of buffalo milk and a concurrent increase in Buffalo Mozzarella PDO demand (fig 1).

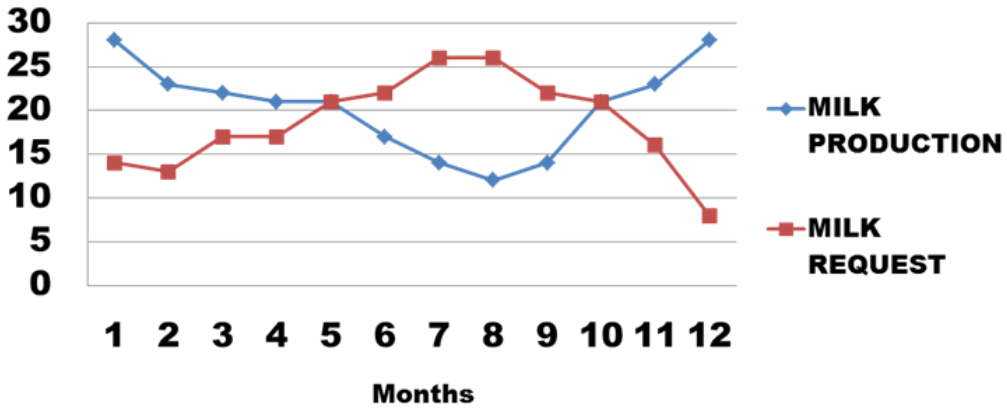


FIGURE 1 AMOUNTS (TONS) OF MILK PRODUCED AND REQUESTED FOR MOZZARELLA CHEESE PRODUCTION DURING THE YEAR

To overcome this inconvenience, producers resort to practices which are not contemplated in the specification. In particular, they freeze milk or curd (mainly curd) in the winter and use them to manufacture Mozzarella when there is a lack of fresh milk in summer. Moreover, some producers buy frozen curd from foreign countries at low costs and mix it with the local curd to produce PDO cheese (Di Luccia et al., 2009).

Mainly, commercial fraud regards use of frozen curd rather than frozen milk, as curd storage and transport are cheaper and easier, requiring smaller spaces and lower energy costs.

Consequently, it is necessary to develop rapid and accurate methods to verify mozzarella quality and authenticity, and protect not only the product value but also consumers from frauds.

Food control authorities require, therefore, analytical methods to discriminate between fresh Mozzarella PDO cheese and Mozzarella produced from frozen intermediates, which are not produced according to the PDO manufacturing process.

Recently, there were many debates between who considers the procedural guideline of PDO unsuitable for the industry and unable to fulfill the huge request coming from the global market and who wants to keep the traditional method of production.

In fact, the use of frozen products could represent a huge source of earnings. A clear scheme was summed up in the local magazine 'Il Test' and reported in fig.2

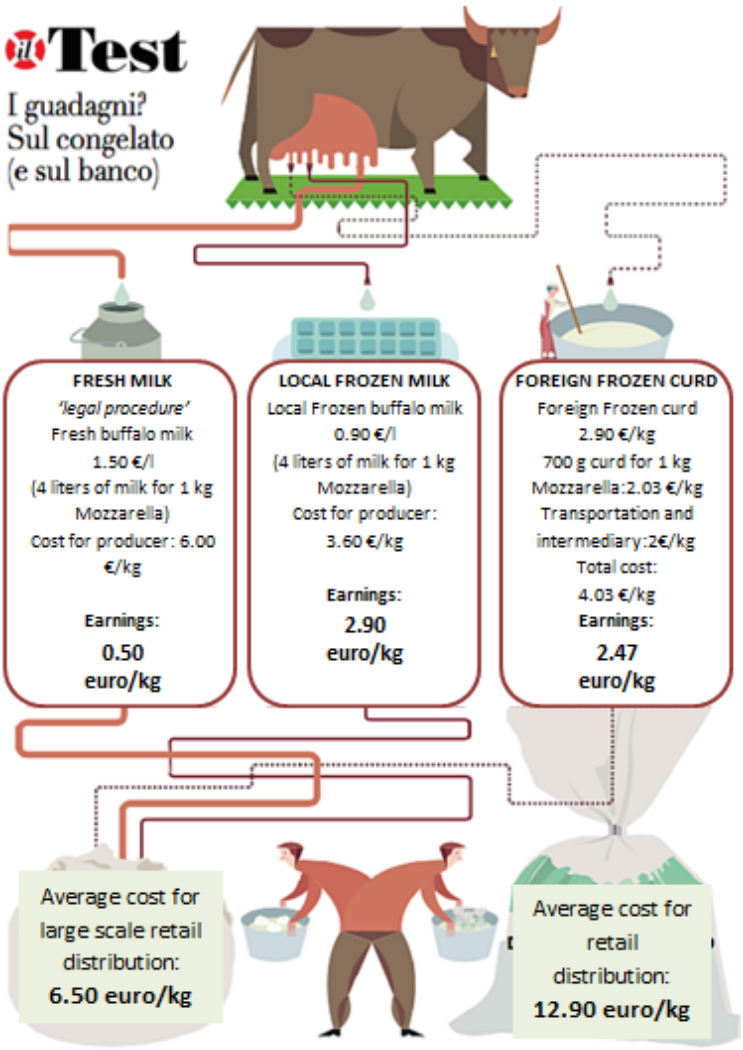


FIGURE 2 EARNINGS DERIVING FROM THE USE OF FRESH OR FROZEN PRODUCTS IN MOZZARELLA CHEESE-MAKING PROCESS

As can be observed, producing mozzarella from fresh milk, producers earn 0.50 euro/kg, while producing the same cheese from frozen milk or curd, the incomes increase to 2.90-2.5 euro/kg.

The earnings indicated in the scheme refer to Mozzarella sold to the large scale retail distribution. Considering sales directly to the final consumer, earnings jump to 6.90 euro/kg in the first case (fresh milk), 9.30 euro/kg when local frozen milk is used and 8.87 euro/kg when foreign frozen curd is used for mozzarella cheese production.

It is important to specify that the freezing of milk or curd is allowed in the case of the non-DOP Buffalo Mozzarella cheese or for *pasta filata* cheese from cow milk (*fior di latte*), while these practices are not allowed in Buffalo Mozzarella cheese with DOP specification (Mucchetti and Neviani, 2006).

Usually, when frozen curd is used, approximately 20-40% of frozen material can be used, since higher percentage may lead to technological and quality problems.

1.2 Background

The application of low temperatures is a widely used system in the food industry for the preservation of many perishable products, though negative effects on food quality cannot be ignored.
Little data is available in the scientific literature about the effects of freezing on buffalo milk and curd.
Some authors examined the effects on proteolysis, lipolysis and sensory characteristics of the frozen curd addition in cheeses obtained from bovine, goat and sheep species (Alonso et al., 2013; Picon, et al., 2013; Pazzola et al., 2013; Van Hekken et al., 2005; Todaroet al., 2011; Zhang et al., 2006). In particular, according to Zhang et al. (2006), good quality sheep cheese can be produced from ovine milk that is frozen at −15°C and −25 °C for 6 months without affecting the cheese yield or composition. Pazzola et al. (2013) suggested that considerable decreasing renneting properties for sheep milk after a long-freezing storage can be obtained, which implies that the freezing of milk should be limited to periods shorter than 5 months. Todaro et al. (2011) reported different effects of freezing according to the species, for example goat milk is more affected by the length of the freezing period and the typology of defrosting than ovine and bovine milk. Van Hekken et al. (2005) found no significant proteolysis during the refrigerated ageing of soft cheese.

Among freezing effects on dairy products, other authors reported an increase in furosine content (1 mg every 30 days) during storage of cow milk at -18°C, a marker that is normally used to recognize the presence of reconstituted milk powder in raw and pasteurized milk (Resmini et al., 1992).

Little data is available about the effects of freezing buffalo milk or curd.
Di Luccia et al. (2009) identified a casein fragment (γ4-CN), which is derived from the action of plasmin on β-casein (Ismail and Nielsen, 2010), as a marker of frozen milk in Buffalo Mozzarella PDO cheese. This fragment was detected as a faint band in fresh milk, and an increase in its intensity was observed during the freezing of milk (-20°C for 12 months). Moreover, recently, a fragment derived from αS1-casein (αS1-I) was also suggested as a possible indicator of frozen curd in Buffalo Mozzarella cheese (Petrella et al., 2015), even if the thermal and time histories of the analysed mozzarella cheeses remained unknown in this study.

Notwithstanding freezing is a widely method used to extend shelf-life of food, many studies in scientific literature report negative effects on food microstructure.
Food microstructure can be defined as the spatial organization of structural components of food and their interactions (Herremans et al., 2013).
Modifications that occur after the thawing process of pasta filata cheese were studied by some authors using Nuclear Magnetic Resonance (NMR) technique and Scanning Electron Microscopy (SEM).
Kuo and Gunasekaran (2009 and 2003) showed modifications of physical characteristic and decreasing of mozzarella quality after defrosting the cheese. Textural implication due to changes in the spatial patterns of protein network, resulting from the mechanical effect of ice formation and chemical effect of higher solute concentrations were detected by Reid and Yan (2004) and Graiver et al.

(2004). Damaging of protein matrix, rupture of water retaining protein fibers due to the growth of the ice crystals were studied by Kuo et al (2001). Changing of water self diffusion coefficient and relaxation time of water were analysed by Kuo et al. (2003).
All these works studied mainly the effects of freezing and thawing of the final product mozzarella cheese.
Different effects can arise when the final product is produced from frozen raw material or frozen intermediates.
Addeo et al. (1992) studied the texture of buffalo mozzarella cheese produced with frozen milk by SEM. They found that the structure of mozzarella produced with frozen milk resulted more compact while a more porous and less compact casein fibers structure was found in mozzarella from fresh milk that was similar to a sea sponge where spaces between casein fibers matrix are occupied by fat and non-structured water.
NMR and SEM techniques used to obtain information on food microstructure are mostly invasive and require sample preparation or are limited to specific applications (Frisullo et al., 2012). X-ray micro-computed tomography (x Ray-mCT) is an innovative radiographic imaging technique that enables non-destructive and non-invasive 3D imaging, and analysis aimed at the internal examination of the structural arrangement of products (Landis & Keane, 2010). The same sample can be scanned numerous times under different conditions. X-ray mCT also enables scanning of the entire sample due to its large field-of-view without any sample preparation (Leonard et al., 2008). X-ray mCT enables samples to be studied in their natural state at atmospheric temperature and pressure. A 3D microstructural investigation of samples in a near-native state and at high resolution is possible.

The study of thermal properties (Differential Scanning Calorimetry - DSC) (Tunick 2015) and the metabolomic approach (Cevallos-Cevallosa, et al., 2009) were also used as effective ways to verify authenticity of food products.
DSC technique was used to discriminate the real mozzarella cheese from mozzarella cheese produced with addition of calcium caseinate to milk, that, if not specified in the label, is an illegal imitation of the product (Tunick and Malin, 1997).
The metabolomic approach is based on identification and quantification of characteristic metabolites of food. Metabolomic identifies a ‘‘molecular fingerprint’’ that accurately represents the food product and discriminates from different or fraudulent varieties (Lindon et al., 2007). The Nuclear Magnetic Resonance (NMR) spectroscopy is recognized as suitable and reliable to contribute to metabolomic studies of food products (Brescia et al., 2005; Gianferri et al., 2007; Kuo and Gunasekaran, 2003; Luykx & Ruth, 2008; Powers, 2009; Sacco et al., 2009; Capozzi et al., 2015).
Practical uses of NMR were demonstrated by some authors, such as *in vitro* study of proteins digestion of aged Parmigiano Reggiano cheese, combining H-NMR and low-field NMR (Bordoni et al., 2011), or the assess of foodstuff quality through identification of biomarkers (Savorani et al., 2010, Shumilina et al., 2015).
Mazzei and Piccolo (2012) used HRMAS-NMR technique to discriminate the geographic origin of Buffalo Mozzarella PDO cheese by a metabolomic approach.

1.3 Aims of the thesis

Currently, the use of frozen milk or curd in PDO Mozzarella cheese-making process is not allowed. Therefore, finding a method that enables their identification in the Buffalo Mozzarella PDO cheese would be an important goal to protect this high-value product.

The food control authorities require analytical methods to discriminate between fresh Mozzarella PDO cheese and Mozzarella produced from frozen intermediates, which is not produced according to the PDO specification.

At the aim, the emerging analytical challenge is to trace the milk and curd history in buffalo dairy products.

Although finding a way to reveal commercial fraud appears critical, it is important to understand if the modifications that may occur during freezing storage involve quality loss.

With the final purpose to evaluate the possibility to trace frozen milk or curd in Buffalo Mozzarella PDO cheese, the project was divided into two phases.

In the first phase, chemical modifications that can occur during freezing storage of buffalo milk and curd were assessed. Firstly, the effectiveness of markers designated in scientific literature as indicators of Buffalo Mozzarella cheese freshness were evaluated.

As previously said, commercial fraud regards the use of frozen curd rather than frozen milk, as curd storage is more convenient in terms of costs and spaces. Therefore, in the second phase, the chemical composition and textural properties of Buffalo Mozzarella cheese produced with addition of frozen curd were evaluated.

2. The Buffalo Milk

Fresh buffalo milk is the only raw material which has to be used for Buffalo Mozzarella PDO cheese. The milk has to be destined to cheese-making process within 60h after milking.

2.1 Buffalo milk composition

Water buffalo milk is defined as the normal lacteal secretion free of colostrum, obtained by the complete milking of one or more healthy water buffalo.
Milk is a heterogeneous, complex mixture, in which three different phases can be recognized:

- *true solution phase*, composed of substances soluble in the water, among which carbohydrates, in particular lactose, mineral salts, soluble proteins, non-proteinaceous nitrogenous substances, enzymes, water-soluble vitamins;
- *colloidal suspension phase*, composed of casein micelles, albumin, globulin dispersed in the water solution, part of phosphate and calcium citrate;
- *emulsion phase*, composed of fat globules in aqueous phase and fat-soluble vitamins.

Environmental and genetic factors caused changes in milk composition over the years. In the last decades of 20th-century, increases in fat and protein content (from 7.3 to 8.3% and from 4.4 to 4.8% respectively) were observed in Italian buffalo milk (Zicarelli et al., 2004a). Differences in milk composition can also due to breeds, management and feeding systems.
The average composition of buffalo milk was reported by different authors over the years (Altman and Dittmer, 1961; Jan, 1999; Ahmad et al., 2008; Menard et al., 2010; Han et al, 2012). The range values of the main milk component are shown in tab. 1

TABLE 1 CHEMICAL COMPOSITION OF BUFFALO MILK

g/100g milk				
Fat	**Protein**	**Lactose**	**Ash**	**Total solids**
7.1-8.0	4.0-5.0	4.6-5.6	0.8-0.9	16.7-17.7

Fat

Milk fat has the most complex structure and composition of all lipids found in food (Tunik, 2015) and it represents the main component of the milk total solid. Varrichio et al. (2007) reported an average value of 8.3% which can also reach the 15% in certain conditions, while Tonhati et al. (2011) found an average value of 9.0 % ± 2.5.

Milk fat is composed primarily of triglycerides (nearly 98% of the total milk fat). Other milk lipids include diglycerides, monoglycerides; phospholipids, cholesterol, and free fatty acids. The triglycerides contain 24-54 carbon atoms, and the position of fatty acids on the glycerol skeleton is not random but it depends on the enzymes involved in their biosynthesis (Tunik 2015). Triglycerides can be classified on the basis of their melting point and molecular weight. Ramamurthy and Narayanan (1974) found 9–12% of high melting triglycerides in buffalo milk fat. Arumughan and Narayanan (1982) reported an high, medium and low molecular weight triglycerides in buffalo milk in percentage of 42%, 17%, and 41% of total, respectively.

The fatty acids composition of buffalo milk can be affected by many factors, such as breed (Talpur et al., 2007), stage of lactation (Arumughan and Narayanan, 1981), health, age (Fox et al., 2000; Qurshi et al. 2010), season (Talpur et al., 2008; Asker et al., 1978), feed (Patiño et al., 2008). Differences in chemical composition may be present not only among species but also within species i.e. among breeds of buffaloes.

Among minor component, a significant correlation has been found between phospholipids, fat content and size of fat globules of buffalo milk. A range of 25.7-29.6 mg/100ml was found in buffalo milk, with a variability during the year.

Moreover, a lower cholesterol amount was found in buffalo milk respect to cow milk (Zicarelli 2004b).

Lipids in raw milk are in form of emulsified globules consisting of a triglycerides core covered with a protective layer, the milk fat globule membrane (MFGM). The buffalo milk fat globule has spherical shapes as that of other milk but differs from fat globules of other ruminants in its rheological characteristic. A volume of 1 ml buffalo of milk contains about 2.7 million fat globules, of which the majority (about 60%) have a size between 3.5 and 7.5 µm (Abd El-Hamid and Khader, 1989; Ahmad et al., 2008). Higher percentage (20.3%) of large fat globules (16-18µm) has been found in buffalo milk but not in the milk of other ruminants. The fat globule in buffalo milk is bigger than in cow milk, with an average size of 5µm and 3.8 µm respectively. El-Zeini et al. (2006) reported an higher average globule sizes in buffalo milk of 8.7µm.

The MFGM of buffalo milk fat globule membrane is composed of about 60% lipids, of which 66–78% are saturated fatty acids, and 22–34 % are unsaturated fatty acids. The 30% of MFGM is composed of proteins.

Proteins

Buffalo milk is also a reach source of proteins, more than cow milk (Ahmad et al., 2008). Of the total proteins, nearly 80% are caseins and the remaining 20% are whey proteins with traces of minor proteins (Laxminarayana and Dastur, 1968; Sirry et al., 1984).

Casein plays a fundamental role in cheese-making processes.

Almost all casein is present in the micellar form (Farrell et al., 2006). Caseins are sub-classified into αs1-, αs2-, β- and k-casein. The amount of each fraction in milk is reported in tab 2 (Pandya and Khan, 2006). Their molecular weights range between 19 KDa and 25KDa.

TABLE 2 CASEIN FRACTIONS IN BUFFALO MILK

Nitrogenous fractions	Concentrations
Total casein	**37.8**
αS1-casein ($g.kg^{-1}$)	16.2
αS2-casein ($g.kg^{-1}$)	2.5
β-casein ($g.kg^{-1}$)	14.2
κ-casein ($g.kg^{-1}$)	4.9

Micelles are aggregates with a porous and spongy appearance, disperse in a continuous phase of water, salt, lactose, whey and protein. Ahmad, 2010 reported a variable diameter of casein micelles ranging from 20 to 300 nm, with an average value of 190 nm. Buffalo micelles are about 10-20 nm bigger than that of cow.
The structure of the micelles is composed of many sub-micelles that present a higher percentage of k-casein on the external surface, since they are more polar than the other fractions. k-casein forms a kind of protective role which allows the micelle to remain in suspension (Farrell et al., 2006).
The sub-micelles are joined together by the presence of phosphoserinic sites, located in the external part of the sub-micelles. The serine amino acid present in protein chains can bond phosphor groups. The four casein fractions have different serine values; for example, αS2 has many residues, determining a higher presence of phosphoric groups. The bonds between calcium phosphate and phosphoserinic groups determine the formation of bonds among the sub-micelles (Schmidt 1982). The aggregation of casein molecules inside sub-micelles is due to several hydrogen bonds, while the total micellar structure is made stable by apatite bonds, made of ionic calcium interacting with the phosphates linked to casein. The role of calcium and the pH are essential to keep the system stable. In fact casein can precipitate by the acidification of milk with a pH of 4.6 (casein isoelectric point) as well as by curd's enzymatic action.
By electrophoretic techniques, concentrations of 44% αs-, 53% β- and 3% k- casein fractions were found in buffalo milk, while 55, 39 and 6% for αs-, β-, and k- casein, respectively were detected in cow milk (Ganguli and Bhalerao, 1964). All three fractions of buffalo milk casein have slower mobility than cow milk casein.
The amino acidic sequence of all casein classes is similar for 95% between buffalo and cow milk.

Buffalo αs1-CN is a polypeptide chain of 199 amino acid residues and a molecular weight of 23.5KDa (Feligini et al., 2009). Amino acid composition of buffalo and cow αs1-casein is similar. Compared to the variant C of the bovine homologous, it differs for the substitution of 10 amino acids that determine its lower negative charge and so its lower electrophoretic mobility through UREA PAGE (pH8.6) (Chianese et al., 1996). Buffalo αs1-CN is more heterogeneous for the presence of at least three phosphorylated components αs1-CN 6P, 7P, 8P, compared to the bovine homologous 8P, 9P. Due to a mutation, there is a phosphorylate residue instead of a hydrophobic one, which highlights the non-polar behavior of a protein trait. In particular the lack of phosphoserine 115 in buffalo αs1 casein reinforces the non-polar character of this protein (Ferranti et al., 1998). Faccia et al. 2014 reported that

the quantification of the αs1-CN fragment deriving from chymosin action can be effective to reveal the use of frozen curd in Fiordilatte (mozzarella cheese produced with cow milk). In fresh cheese such as mozzarella, primary proteolysis of αs1-CN and β-CN has been reported during the refrigeration storage at 4°C (Farkye NY et al. 1991; Di Matteo et al. 1982). The family of αs1 and αs2 casein differs in the amino acid sequence. The αs1-CN are particularly sensitive to calcium at all temperatures.

Buffalo αs2-CN is made of 207 amino acids. It is bound to a different number of phosphate groups (Ginger et al. 2002) that, during electrophoretic separation, make it move between αs1-CN and β-CN. It is weakly hydrophobic and this separates it from other types of calcium-sensitive casein. Its molecular weight varies from 25,150 to 25,390 Daltons because of the different phosphoric residues (Salvadori del Prato 1998).

Buffalo β-CN is a single polypeptide chain, whose primary structure is made of 209 amino acids, like its bovine counterpart. It has a molecular weight of about 24kDa (Feligini et al., 2009), similar to αs1-CN (Corradini, 1995). It's more hydrophobic due to the presence of proline and non-polar α-amino acids. There are 9 known genetic variants, of which Variant B is more common in the Mediterranean race, bred in Campania. β-CN is the preferential substrate for plasmin, an endogenous enzyme of blood origin, derived from milk (Eigel et al. 1984; Fox et al. 1994). Medical conditions, such as mastitis, enhance the activity of this enzyme, whose action determines the formation of 4 fragments (f) designated as γ1, γ2, γ3 and γ4-CN. Plasmin cleaves preferentially Lys-X peptide bond. The hydrolysis of the Lys28-Lys29, Lys105-His106and, Lys107-Glu108 and Lys68-Ser69 bond lead to the formation of the f(29-209) γ1-CN, f(106-207) γ2-CN, f(108-209)γ3-CN and f(69-209)γ4-CN (Alais 1984, Trieu-Cuot & Addeo in 1981). The fragment γ4-CN resulted present in buffalo milk and absent in bovine milk. Apart from plasmin, β-CN can be hydrolyzed by chymosin, exogenous enzyme deriving from the rennet, also active on αs1-CN (Carles and Ribadieau-Dumas, 1984).

Buffalo κ-CN is a chain of 169 amino acids, like its bovine counterpart, with a molecular weight of about 19kDa. It stabilizes casein micelles present in milk against calcium: it has therefore a colloidal-protector function. The k-CN is a glycosylated protein which is similar as for cow milk. The presence of glycosylated groups at the periphery of casein micelles gives the charge to the micelles (Ahmad, 2010). κ-CN is specifically hydrolyzed into two fragments by chymosin action, by a specific action on the Met105-Phe 106 bond, forming insoluble para K casein (N-terminal fragment 1-105) and macropeptide-casein (C-terminal fragment 106-169). It is the only soluble casein in the presence of calcium ions at any temperature.
Caseins represent the 80% of total proteins. The remaining 20% are whey proteins. The protein components of whey include serum albumin (SA), a-lactalbumin (a-LA), b-lactoglobulin (b-LG) and immunoglobulins (Fox, 1989). Besides, the minor proteins include lactoferrin, lactoperoxidase and lysozyme, which have important antimicrobial and carrier functions (Marshall, 2004; Parodi, 2007). Among whey proteins, β-lactoglobulin (β-Lg) and α-lactalbumin (α-LA) are the most abundant. Quantitative data about whey proteins from Mediterranean water buffalo (the milk used to produce the PDO Mozzarella cheese) showed concentration of 4.0 and 2.5

g/l for β-Lg and α-LA respectively (Buffoni et al., 2011). Whey proteins are well known for their high nutritional value. The amino acid composition of buffalo β-lactoglobulin (β-Lg) is identical to that of cow milk (Mawal et al., 1965). The molecular weight of buffalo b-lactoglobulin was reported as 18,266 Da, while a-lactoalbumin as 14,236 Da (Buffoni et al., 2011) and no genetic polymorphisms have been observed (Malik and Bhatia, 1977).

Buffalo milk is also a rich source of lactose, more than cow milk. Lactose is a disaccharide made up of glucose and galactose. Lactose makes a major contribution to the colligative properties of milk, such as osmotic pressure, freezing point depression and boiling point elevation (Varman and Sutherland, 2001). With heating, the milk undergoes to the Maillard reaction: the lactose binds to the amino groups of lysine residues of the milk proteins to form, firstly, a Schiff Base or a lactosylamine, which is further converted to the Amadori product lactulosylamine. The formation of early Maillard products leads to a considerable loss of bio-available lysine (Mauron et al., 1990 and Birlouez-Aragon et al., 2004). As a consequence, the nutritional value of milk proteins can be largely decreased.

Among minor compounds, buffalo milk has been found to contain more minerals than cow milk. The content of minerals has been reported by Cashman (2002a, b). Buffalo milk has a high calcium content. Most of calcium is found in insoluble form (67.6-82.6% of the total calcium). Ahmad et al., 2008 report a content of micellar calcium of 1.12 mM/g casein. The ionizable calcium of buffalo milk represents 34.6% of the soluble calcium. Buffalo milk is also rich in phosphorous contents. The phosphorous is in the dorm of colloidal inorganic phosphate (42.4% of total), soluble inorganic phosphate (30.0%of total) and esters phosphorous (9.2% of total) (Abd El-Salam and El-Shibiny, 1966).

2.2 Physical-chemical properties of buffalo milk

Buffalo milk is characterized by a very white color. The pH ranges from 6.57 to 6.84 and is not influenced by month, lactation number, or season, but correlated with solid-not-fat and lactose contents (Minieri et al., 1965). Acidity was reported as ranging from 0.17-0.26% (Mahmood and Usman, 2010). The freezing point of buffalo milk was found in the range of – 0.552 to – 0.558° C (Hofi et al.,1966) while the refractive index (at 40°C) from 1.346 to 1.353

2.3 Enzymatic activity in milk

Milk contains numerous minor proteins having physiological effects. These minor proteins include a wide range of enzymes (Fox, 2001).

Lysozyme: Lysozyme (LZ) is a basic protein enzyme with a low-molecular weight and important component of the antibacterial system in milk. Priyadarshini and Kansal (2002b) found the molecular weight of buffalo milk LZ to be 16 kDa, and determined its antibacterial activity. Buffalo colostrum contains five times more LZ activity than mature milk (Priyadarshini and Kansal 2002a) and higher specific activity than that of cow milk LZ. Buffalo milk LZ is active over a wide range of pH

and its activity is strongly influenced by the molarity of the medium. LZ activity in buffalo milk was not influenced by parity and stage of lactation; however, it increased during extreme weather conditions in winter and summer. The higher LZ activity in buffalo milk possibly is one of the factors responsible for lesser incidences of udder infections in buffaloes. LZ has found application in food preservation like egg-while lysozyme is already being used successfully as an antimicrobial in many foods, especially in cheese (Benkerroum, 2008). LZ in buffalo milk is more stable than in cow milk during storage and heat treatment (Priyadarshini and Kansal, 2002b).

Lactoperoxidase: Buffalo milk lactoperoxidase (LP) has been studied extensively (Van Nieuwenhove et al., 2004). LP has antimicrobial properties and because of its broad biocidal and biostatic activity, LP has found many commercial applications, especially targeting oral pathogen (Tenovuo, 2002).
Enzymes play also an important role from a technological point of view.
Some enzymes are important indicators of heat treatment (alkaline phosphatase) or healthy conditions of milk.
Enzymatic proteolysis can have positive effects on aspects such as the development of flavor or textural changes during cheese maturation. However, an undesired proteolysis can negatively affect the quality of milk, milk products and their shelf-life.

Proteolytic enzymes
Some proteases in milk come from the contamination of microorganisms and other are transferred into milk from animal blood.
Proteolytic enzymes in milk are divided into endogenous, such as plasmin, cathepsin D, cathepsin B and elastase, and exogenous, such as chymosin, pepsine and microbial enzymes accidentally introduced, or introduced during the technological process.
Proteases contained in milk degrade proteins, reducing them into small fragments.
Alkaline and acid proteases can be found in the milk. Alkaline proteases have a molecular weight of about 48,000 Da and an optimal pH action of 7.5. Plasmin belongs to this group. Its inactive form is zymogen, whose activation occurs thanks to the plasminogen action.
Acid proteases, instead, have an optimal activity at pH of 4.0 and a molecular weight of about 36,000 Da. They are especially active on αs1-CN, producing αs1-I-CN fragments. Chymosin belongs to this group.

Chymosin is the main enzyme responsible for the primary proteolysis in most cheese varieties. This aspartyl protease is contained in the rennet together with pepsin; the main target of chymosin is peptide bond Phe105-Met106 of the κ-CN fraction, cleavage of which determines milk coagulation and curd formation (Petrella et al., 2015). Chymosin is active also on both αS1-CN and β-CN in solution (Carles and Ribadeau-Dumas, 1984) but in cheese it appears to hydrolyze mainly αS1-CN (Fox, 1989; McSweeney et al., 1993; Mulvihill and McCarthy, 1993; Scherze et al., 1994; Sienkiewicz et al., 1994). The primary site of chymosin action on αs1-CN is Phe23-Phe24, yielding the fragment αS1-I-CN f(24–199) (Hill et al., 1974; Carles and Ribadeau-Dumas, 1985; McSweeney et al., 1993).

Faccia et al. (2014) reported that quantification of the fragment αS1-I-CN can be effective for revealing the use of stored curd in high-moisture cow mozzarella because chymosin is denatured by the high temperature (90°C) used during the stretching phases, it results in a low level of αSI-CN hydrolysis (Hayes et al., 2002; Sheehan et al., 2007; Faccia et al., 2014). Chymosin also cleaves αs1-casein at Phe28-Phe29, Leu40-Ser41, Leu149-Phe150, Phe153-Tyr154, Leu156-Asp157, Tyr159-Pro160, Try164-Tyr165, Leu11-Pro12, Phe32-Gly33, Leu101-Lys102, Leu142-Ala143, and Phe179-Ser180, with the cleavage rate depending on the ionic strength and pH (Mulvihill and Fox, 1979; McSweeney et al., 1993).
Starter bacteria enzymes are known to affect secondary proteolysis (Yun et al., 1995; Gagnaire et al., 2004).
Primary proteolysis in mozzarella cheese is due to the incomplete thermal inactivation of enzymes during the stretching process (Richardson, 1983; McGoldrick and Fox, 1999); the production of αS1-I-CN takes place in the earliest stages of production and proceeds at a rate that depends on curd cooking temperature and water activity (Tunick et al. 1993; Yun et al., 1993), on the amount of the residual rennet (Dave et al. 2003), as well as on pH and NaCl concentration (Feeney et al., 2002). Even though the primary proteolysis rate can be affected by several factors, it progresses quite slowly in both low- and high-moisture mozzarella (Kindstedt et al., 1988; Tunick et al., 1993; Faccia et al., 2014).

Plasmin (PL), the main indigenous milk protease , is present in blood and milk in the form of a non-active precursor, plasminogen (PG), and after the action of the plasminogen's tissue activator (tPA) and PG urokinase activator (uPA), is transformed in its active form. PL is made inactive by antiplasmin-alpha 2 and by serine protease inhibitor (serpin).
PL, PG and PG activators are associated to casein micelles, while the inhibitors are located in the whey.
PL has an optimal action at pH 7.5 and temperature of 37°C. Kelly and McSweeney, 2003).
Considering its physiological role in blood, it is not surprising that the activity of plasmin is part of a complex system of activators and inhibitors (Ismail B & Nielsen SS. 2010). PG is not present in mammary glands and consequently, in milk; the origin of PL and PG is blood (Alichanidis et al. 1986); the concentration of plasmin in milk and blood is about 0.3 and 200 mgL-1 respectively (Halpaap et al. 1977).
PL starts working when it is activated, even if it is present in blood in a non-active form(PG).
The concentrations of PL, PG and plasminogen activators (PA) are low in the first stage of lactation (Bastian et al. 1991a). Nevertheless, the concentrations of such enzymes in milk increase with the later stages of lactation (Bastian et al. 1991a) and are higher during the dry period of the animal. The activity of plasmin in milk is higher in older animals and increases with the lactation number.
Plasmin activity is also influenced by mastitis (Politis et al. 1989), as the increase in the number of somatic cells (White et al.1995, Heegaard et al.1994) contributes to raise the number of PA that lead to the production of its active form, PL.
Plasminogen converts plasmin faster at body temperature compared to refrigeration temperature. Pasteurization at 72°C for 15 seconds reduces the activity of plasmin in milk only by 10-17% (Bastian & Brown, 1996). However, the

storage of pasteurized milk leads to an increase in plasmin activity due to the inactivation of active inhibitors of plasminogen present in milk. To completely inhibit plasmin during storage it is necessary to heat milk at 142°C for 18 seconds or 120°C for 15 minutes.
Kaminogawa et al. (1972) suggested that plasmin may be transported from plasma across mammary epithelial cells.

Plasmin activity is influenced by many factors.

- Mastitis: Zachos et al., 1992 and Politis et al. 1989 a/b reported that Plasmin activity is higher in mastitic milk than in normal milk. They concluded that even after curing mastitis, plasmin activity does not return to its pre-infection level. This may be one reason why milk from older cows has higher plasmin activity than milk from younger cows.
- Lactation number: Milk from older water buffalos has higher plasmin activity. There is an interaction between age and stage of lactation. Plasmin activity increases in milk from older subjects during lactation (Politis et al. 1989 b).
- Stage of lactation: Plasmin activity and plasminogen increase at the end of lactation (Politis et al. 1989 ab, Bastian et al. 1991b). Total enzyme (PL and PG) increase during the first six months of lactation, but plasmin in the last three months increase drastically.
- pH and temperature conditions: Plasmin is active at pH 7.5 – 8.0 and 37°C (Fox et al. 1981).
- Storage conditions: The PL activity is active during cold storage (2-5 °C). The β-CN becomes more soluble at lower temperature (Crudden et al. 2005).

Other authors reported an increase in plasmin activity in milk, curd and mozzarella cheese when cold stored at 4°C (Somers JM. et al. 2002).

- Several studies have shown that bacterial proteases affect the plasmin system. Plasmin activity has been reported to decrease with microbiological growth during storage. Decreased plasmin activity was observed in fresh raw milk after 4 day of storage at 4°C. Further studies have shown that some bacterial proteases can enhance the activity of PA, such as Pseudomonas fluorescents protease enhances PA activity by increasing its catalytic activity.

Bacterial proteases

Among numerous factors that can contribute to proteolysis in milk, bacteria proteases play an important role. Gram-negative psychrotrophs are responsible for spoilage, in refrigerated raw milk.
The proteases produced by the psychotropic bacteria can destabilize the casein micelles by hydrolyzing κ-casein (Mitchell and Marshall 1989, Cromie 1992), resulting in low cheese quality (Gruffery & Fox, 1988; Champagne et al. 1994).
In particular, Pseudomonas spp. are predominant in milk after pasteurization.

2.4 The micro-organisms of milk

The micro-organisms that are found in milk can come from different sources: the animal, feedstuffs, milking, storing (period of time between milking and cheese-making), environment and man.

In the specific case of milk destined to the production of cheese it is possible to distinguish dairy (useful, necessary, essential) and anti-dairy micro-organisms (that disturb the cheese-making process).

Dairy micro-organisms

The leading factors in the cheese-making process are the lactic bacteria. They are necessary for the lactic fermentation: they transform lactose (the milk sugar) into lactic acid; this acidification process helps the rennet's work, the draining of the whey after the curd has been broken and the cheese maturation. Lactic bacteria are indispensable for the production of buffalo Mozzarella cheese. This acidity is "created" by the lactic bacteria in the milk of origin. If the milk or the curd do not have the right degree of acidity the liquid mass will not string and it would be impossible to produce buffalo Mozzarella cheese.

Anti-dairy micro-organisms

Coliform and butyric bacteria in milk means little attention has been given to hygiene during the process (even during the milking phase). The latter, on the other hand, come from the feedstuffs, the soil or the water and cause cheese to blow.

3. The Buffalo Mozzarella PDO Cheese

3.1 The 'Protected Designation of Origin' specifications

3.1.1 Birth of the Consortium

Campana Buffalo Mozzarella cheese was awarded the prestigious Protected Designation of Origin (PDO) status in 1966. This award recognized the organoleptic and merchandise qualities of this cheese that depends on the environmental conditions and the traditional working methods existing in the specific area of production.
Buffalo Mozzarella PDO cheese represents the most important PDO brand in central-southern Italy and is the fourth at national levels and the third amongst the Italian PDO cheeses.
The Consortium is the only organization acknowledged by the Department for Agricultural, Food and Forestry Policies for the protection, safeguarding, improvement and promotion of this extraordinary cheese from Central and Southern Italy that is loved throughout the world.
The Consortium was created in 1981 to protect the production and commercialization of this particular cheese and defend the Designation of Origin in Italy and abroad. It also aims to encourage the continuous improvement in the Mozzarella's production methods and the consequent qualitative improvement of its production.
The product specification has been frequently updated, and it will further change in the future to make this product always up to date and available worldwide.
In the 1993 the name "Buffalo Mozzarella" was changed in "Campana Buffalo Mozzarella", and then in "Campana Buffalo Mozzarella PDO" with the Regulation (CE) 1107 of June 12, 1996.
In the 1993 the area of origin of milk was established; ten years later, in 2003, the Consortium asked to widen the area of origin with the decree of the Ministry of Agricultural and Forestry Policy (MIPAF) of September 18, 2003.

3.1.2 Production areas

The buffaloes have to belong to the Mediterranean race exclusively and bred in the PDO area, as the Specification requires.
Nowadays, the source areas of the buffalo milk, its transformation into the cheese "Campana Buffalo Mozzarella" include territories in Campania Region (in particular the provinces of Benevento, Caserta, Naples and Salerno), Lazio, Apulia and Molise Region. The complete list of the territories is reported in the PDO specification. These municipalities have been recognized for their link to the area and the tradition in producing Buffalo mozzarella.

3.1.3 The technological process

The Buffalo Mozzarella cheese has the PDO status under European Union regulations. It implies that any cheese-making practice must comply with the rules established by the National Standard for cheese production. For this cheese fresh buffalo whole milk, with a minimum fat content of 7.2% and a minimum protein content of 4.2%, is required.
The milk must be delivered and processed in cheese within 60 hours after milking.
Milk thermization or pasteurization are optional.

The cheese-making process is shown in fig. 3

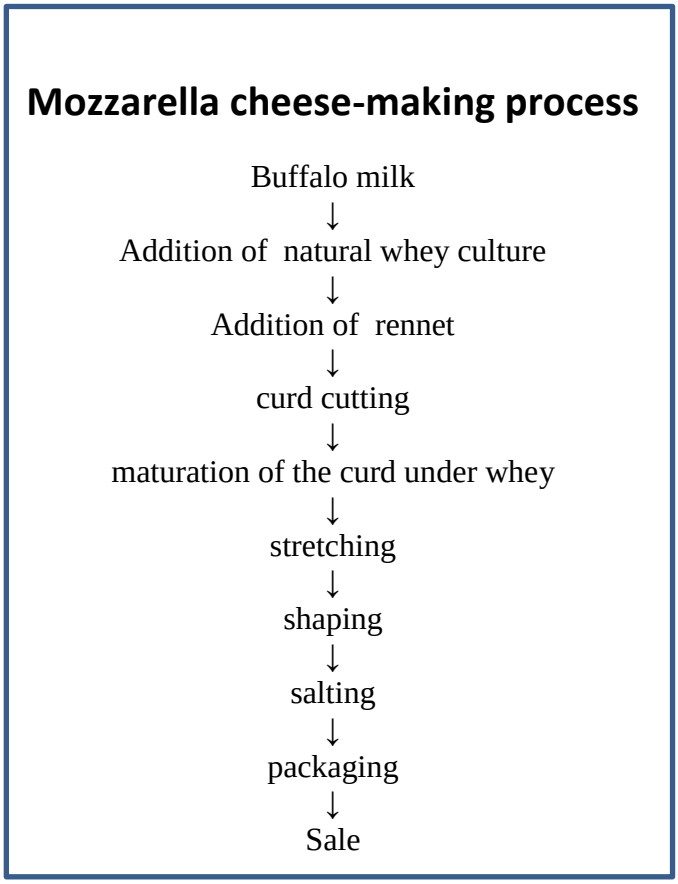


FIGURE 3 BUFFALO MOZZARELLA PDO CHEESE-MAKING DIAGRAM

- ✓ *Acidification and coagulation*

Coagulation is preceded, having brought the milk up to a temperature of 33 - 39°C, by the addition of natural whey that is collected from the same cheese-making processes of the previous day. This addition activates the milk for the following coagulation that takes place in steel containers, after the addition of the exclusive natural calf rennet.

- ✓ *Breakup and maturing of the curd*

After a few minutes, when the milk coagulates due to the intervention of the rennet, the matrix is broken to the size of a walnut. This is the moment when the separating out of the solid and the liquid stages of the milk begins (syneresis). The solid part, called curd, is left to acidify in the serum until is considered as 'mature' or 'ready' for the test of stretching process.
The duration of the acidification of the curd under the whey is one of the variables more sensibly affecting the quality of the cheese. Many factors contributes to the length of this phase, such as the microbial quality of milk, acidity, enzyme concentration, temperature, degree of crumbling of the curd, the quality of the whey rennet used, the availability of calcium. Therefore the time the curd takes to mature can be variable in different dairies or even in the same one. Mozzarella whey, differently to that obtained in the making of pressed cheese, does not undergo any thermal treatment, therefore a selection of indigenous microflora only takes place. Microorganisms inhibiting cheese-production can also develop.
The PDO Specification requires that the maturation of the curd has to take place exclusively by natural fermentation, so without external intervention on the pH by adding organic (citric, acetic, lactic) or mineral (phosphoric) acids to milk before the curd coagulates.
Once the curd is mature, it is minced to prepare it for the stretching phase.

- ✓ *The spinning test*

This empirical test consists in the addition of boiling water to around half a kilogram of broken up curd. Once this has been amalgamated until it has fused, it is stretched with a wooden stick. If it stretches uniformly without breaking, it is considered 'ready' for the subsequent spinning phase. The correctly matured curd is placed on draining tables where the all the residual serum is eliminated.
The thickness of the curd should be less than 20 cm, in order to obtain a quick and homogeneous acidification and to allow faster syneresis.
The syneresis is a spontaneous process, which takes place because of the contractions of fibrils in the proteinaceous graticule. This contraction exerts a pressure on the whey imprisoned in the coagulum graticule, and forces it to leak. The leak of whey determines a narrowing of the net around the globules of fat, and the remaining whey fills the spaces among the globules of fat and the casein net, working as a lubricant (Addeo et al., 1996).
The curd is then cut into strips and placed in special containers where boiling water is added. The contact between the boiling water and the curd causes them to fuse together and the solid form is continually lifted and pulled until it becomes a single, homogeneous mass.
The stretching is the typical phase that makes Mozzarella a pasta filata cheese. Traditionally the pasta is stretched by hand. However, many companies nowadays use automatic machine based on the movement of screws rotating in opposite direction, to knead and stretch the curd which has been previously matured.

✓ *The shaping*

A machinery called 'shapers' produce pieces of a pre-established weight.
The shapes and sizes are defined by the PDO specification. The shapes can vary from the round one to “cherries”, “pearls”, “braids”, “knots”.
The sizes can vary from 10 to 800 g in relation to the shape; the “braid” can weigh up to 3 kg.
After the cheese is formed, it is first left in tanks filled with cold water, to guarantee an initial and important solidification which is then completed in further tanks containing a saline solution (from 10 to 18%) that gives the product the correct level of tastiness. The duration of this operation varies depending on the dairy, but generally it does not last more than 10 hours, for sizes of 400-500 g. During this time salt penetrates into cheese by diffusion, and the speed depends on the saline concentration, on the temperature and the size of mozzarella.
The product is take out from the water bath and immersed in the protective liquid, where the salt concentration tends to re-equilibrate: from the external layers, where the concentration is high, salt moves towards the internal layers. The protective liquid is usually made of an acidic solution containing 2-3 % sodium chloride. Since the salt concentration on the surface is higher than in the protective liquid, a migration process of salt from the product to the protective liquid occur, and so it is possible that during the conservation of cheese in the protective liquid the salt level decreases too much, causing a sloughing of mozzarella.

✓ *The packaging*

Finally the product is packaged in a protective liquid composed of stretching water, salt and diluted acid whey.
The PDO Mozzarella, by law (D.l.vo 109/92, art. 23) can only be sold if pre-packaged at place of origin (heat sealed bags, trays, cups, etc.)
Each package must bear the following information: - the entire regulations for the denomination of Campana Buffalo Mozzarella; - presence of the logos in fig.4

FIGURE 4 LOGOS ON BUFFALO MOZZARELLA PDO CHEESE PACKAGING

3.1.4 Product characteristics

The Specification regulates the shape and organoleptic characteristics of buffalo mozzarella.

External appearance: porcelain white color, ultra-thin crust (about 1 mm), smooth surface which must never be slimy (caused by microflora) nor flaky (caused by incorrect storage). The surface presents, more or less clearly, some irregular features caused by the manual or mechanical detachment of mozzarella from the cheese pasta.
The pasta must have a leaf-like structure, with the external film protecting the underlying pasta from which it must detach with a clean break. The pasta must be slightly elastic in the first 8-10 hours of production and must tend to turn into a melt-in-the-mouth texture, devoid of flaws such as stains, produced by foreign or gaseous fermentations. It must be devoid of dye, preservatives or inhibitors. The texture felt in the first moments of chewing can vary from elastic to rubbery, depending on the hardness of the pasta. When sliced, it releases a whitish, fat whey which smells of lactic starters. The taste is sweet, slightly acerbic, delicate and characteristic, due to the strong aromas of the original milk which depends on the zootechnical feed of aromatic herbs or silage, depending on the season.
The final product has a minimum fat content on dry matter of 52% and a maximum moisture content of 65%.

3.1.5 Safety policy

The control of the finished commercialized product is of particular importance for the Consortium.
The Inspection Department of the Consortium covers the entire national territory and works by means of a control agent with the qualifications of a police role, who takes samples of retail products and analyses them in the laboratories of the Central Fraud Protection Inspectorate of the Department of Agricultural, Food and Forestry Policies.
It must, furthermore, record any irregularities for the area concerned, issue sanctions and/or administrative withdrawals of products and/or their packaging.
Certifications are a guarantee of quality for both producers and consumers, in terms of safety, genuineness and freshness.

3.2 Buffalo Mozzarella PDO cheese in numbers

The buffalo dairy industry has a great economic value, with a production of approximately 41,300 tons of Campana Buffalo Mozzarella PDO cheese in the 2015. This sector had a growing trend during the last years, with an increase of 8,5 % in 2015 respect to 2014. The 25% of this production was exported, in particular to France, Germany, United Kingdom, United States of America, Switzerland and Spain. The number of registered buffaloes amounts to 345,000. In 2015, the sector registered 1371 buffalo PDO farms, and a total of 102 certified dairies. The final consumer price of Buffalo Mozzarella PDO cheese recommended by the Consortium is approximately 10 euro/Kg, but it can be largely affected by other factors, for example transportation and distribution costs (Data provided by the Buffalo Mozzarella PDO cheese Consortium).

4. The freezing of food

4.1 Freezing food technology

Freezing is one of the most important unit operations in food processing and preservation. Among all food preservation methods, it is usually considered the best technique for long term preservation of high quality foods. Properly frozen products are considered by consumers to be closest to fresh foods. Nevertheless, various physical, chemical and biochemical processes take place during food freezing, frozen storage and thawing that are of concern to manufacturers and consumers.

All foods have complex composition and microstructures. During freezing, heat is removed from the material, the water and perhaps other components such as some oils and fats crystallize out, and there is movement of water and other molecules on various scales.
Smaller, thin product will cool and release heat more quickly than large, thicker products. In all cases the rate of heat release will not be constant. The maximum rate of heat release will occur in the initial stages of cooling when the temperature difference between the surface of the product and the refrigerating medium is highest. As the surface temperature of the food approaches that of the refrigerating medium the rate of heat release will be very small.
The faster the cooling, the more nuclei of ice will form, leading to large number of small crystals. The formation of a large number of small intracellular crystals will ensure that the cell is not distorted or dehydrated, and so quality may be improved. If the freezing rate is slightly reduced, only one or a few large intracellular crystals will form. There are reports, however, that at some intermediate freezing rate where a single large crystal forms in the cell, the crystal may cause excessive distortion and rupture the cell, causing excessive drip on thawing. At a faster rate still, crystallization does not have time to occur inside or outside the cell. Thus the tissue does not separate into ice and non-ice phases, but becomes a glass. This is the best situation for preserving viability or food quality; unfortunately this freezing rate can only be obtained in extreme cases (very small samples in liquid nitrogen) and is unlikely to be obtained in industrial food freezing. Furthermore, even if it can be done, much of the food could be undone during thawing, as crystals may form during that stage.
The main factors affecting food quality are:
- Macro-scale water migration: during air freezing, water will evaporate from the surface of the food into the air, because the surface is warmer than the air and therefore its vapor pressure is high. During immersion freezing, water may diffuse into the food or out of it depending on the solute concentration, i.e. water activity, of the water.
- Freeze concentration effect: as the water freezes, the remaining solution becomes more and more concentrated in solutes. The increased concentration may have significant effects on some fresh foods, since high solute concentration may denature the cells of fresh foods. This denaturation is faster at higher temperatures (Arrhenius law), and therefore it is worsened by slow freezing since the food spends

longer at high temperature and high solute concentration. This effect will also occur during thawing.
- Physical effect of ice formation on the microstructure: ice formation may cause distortion, cell wall detachment, cell rupture.
- Large scale mechanical effect of ice: upon crystallization, water expands by around 9%, causing very high stresses and in some case cracking.
- Osmotic dehydration effect: loss of water from the cells, causing distortion of the cells, high drip rates on thawing due to insufficient resorption.
- Lethal effect of intracellular ice (if any) on living cells. Of course, different foods respond in different ways to freezing. Thus with non-cellular foods such as bean curds (tofu) the problem is not the cellular rupture but only the freeze concentration and consistency (Pham, 2008)
The main food alterations are visible when the food is thawed and water syneresis occurs. Water affects foods at several levels such as safety, stability, quality, and physical properties.

4.2 Milk freezing: influence on chemical component

Fat fraction

The fat fraction in milk and most other dairy products exists in the form of an emulsion. The fat globules are coated with a protein and phospholipid membrane after secretion from the mammary gland. Homogenization greatly increases the number and surface area of the fat globules present, and milk proteins quikly adsorb homogenized fat globules to reduce their interfacial tension. During freezing, the physical defect of greatest concern related to the fat phase is the loss of the emulsified state and the separation of the fat phase. Coalescence of the fat during static freezing is caused by mechanical damage to the fat globule membranes by the expanding ice crystals, and the degree is closely related to the extent of freezing. Homogenization before freezing greatly overcomes the de-emulsification of the fat globules in milk because of their smaller size and enhanced surface layers, making them less prone to rupture, although some fat coalescence can still be evident, dependent largely on the rates of freezing and thawing.
The lipid fraction is affected by freezing conditions. A progressive loss of fat in the cheese produced with increasing percentage of frozen milk was detected with a consequent increase in fat in the spun water. The amount of fat that is lost corresponds to what emerges during the thawing step because the fat fails to be incorporated into the structure of the subsequent processing stages. This is definitely a problem of a technical nature and not an alteration of the frozen milk. Some technological expedients, for example the use of microwaves in the thawing process, could serve to minimize this drawback which does not seem to significantly affect the organoleptic characteristics of the Mozzarella, but that may have an impact on the yield of processing (Addeo *et al*., 1992).

Milk solid-non-fat fraction

Freezing and frozen storage can have a large effect on the proteins of milk, causing the casein micelles to lose their stability and precipitate on thawing. This manifests itself either as thickened product or as flocs of casein evident either on the sides of thawed glass or plastic containers or as a precipitate at the bottom. The flocculation of casein from frozen milk is initially reversible with heat and agitation, but becomes irreversible with continued storage. Slow freezing has been reported to result in greater protein stability than fast freezing, possibly related to the effect of rapid freezing in promoting lactose nucleation. The stability of casein in frozen milk depends heavily on the state of lactose (Douglas Goff, 2006)
Substances that are soluble in water, such as lactose and salts, increase their concentration in the not frozen portion modifying the equilibrium between the soluble and the insoluble fraction. The conditions resulting from the formation of ice and from the crystallization of lactose (pH fall, increase in the saline concentration, dehydration of the proteins) affect the dispersibility of the proteins and, in particular, of the casein, causing their flocculation, which is function of the intensity of the 'freezing'.

4.3 Freezing of dairy products

Frozen dairy products can be divided into two categories: (1) products frozen to increase their shelf life and thawed before consumption or further processing and (2) products in which the freezing process is responsible for the development of the desired structure and texture and which are consumed in the frozen state.
Dairy products vary considerably in their moisture content, from 87-91% for whole and skim milk to 3% for milk powder. Obviously, products with higher water contents will involve significantly more physical alterations during freezing than those with lower water contents. Although milk can be frozen for preservation or extended shelf life, its high water content makes this process somewhat economically unattractive, and suggests the need for concentration prior to freezing- the flavor of dairy products after freezing and thawing generally are comparable to their fresh counterparts under normal circumstances, with lipid oxidation being the greatest concern. However, the physical effects of freezing may be quite noticeable upon thawing. While flavor deterioration, if present, cannot be rectified, physical changes that may have occurred during storage may be reversible if the thawed product is to be pasteurized and homogenized for further processing by heating or homogenization into dairy or other food products.
There is a commercial interest in increasing the effective storage life of cheese in the marketing chain. Cheese preservation through freezing technology appears to be a good candidate for extended storage, as low temperature slows down and/or prevents changes during the cheese ripening period (Johnston 2000). Frozen storage has been reported as the technological treatment that can provide the longest, highest quality shelf life, in comparison to many other preservation technologies (Reid 1990).
Beside benefits, it should be considered that the freezing technology, and the subsequent thawing process, can be also cause of textural and appearance defects

not acceptable to consumers. Johnston 2000 reported a poor cohesiveness, a watery surface, fat leakage, acid flavor and discoloration in Mozzarella cheese.
The change of water into ice, caused by the lowering of temperatures, has two main consequences: the mechanical consequences of the formation of ice and the chemical consequences of the increased solute concentrations in the unfrozen aqueous phase.
During heat progressive removing, ice is first nucleated at the surface of the cheese; then an ice front forms and moves slowly through the cheese towards its center. The structure of this advancing interface depends upon the rate of heat removal, the internal temperature gradients within the freezing material and the geometric shape of the material. Because the ice phase consists of pure water, the solutes in the aqueous phase such as ions, sugars, acids are rejected from the advancing ice fronts, forming regions of higher concentration ahead of the front and in dendritic inclusions between the growing ice crystals. This freeze-concentration process is associated with changes in pH, ionic strength and osmotic pressure, which can trigger denaturation of structural cheese proteins. Therefore, the mechanical consequences of freezing are directly related to the spatial patterns of ice formation, while the chemical consequences are related to the degree of solute redistribution (Reid, 2004).
Freezing conditions can affect the effects of the treatment on the product, such as the spatial patterns of ice formation and, therefore the spatial patterns of solute distribution within the frozen object (Reid 2000a).
Because cheese structure can be considered as a protein network embedded with a dispersed phase of fat globules, minerals and water, changes in the spatial patterns of the cheese protein network, resulting from the mechanical effect of ice formation and the chemical effect of higher solute concentrations, may have significant textural implications Reid, 2004.
However it is important to distinguish the freezing of dairy products for the consumer market and the freezing of dairy products destined to further processing.

4.4 Influence of freezing on the microbial flora

Lowering the temperature of food below the freezing point can cause damages to the microbial cells. At freezing temperature (<-18°C) no microorganism is able to grow. Freezing causes a decrease of the water activity (-18°C = 0.841) and an increase in concentration of solids which influence the activity of the microorganisms.
During the freezing, part of the microbial population undergoes irreversible damage due to the formation of ice crystals which cause an increase in the cell volume and a consequent breaking of the wall that bring to the lysis of the cell. Many microorganisms can survive during freezing process and this resistance depends on a number of factors. In general the Gram-negative bacteria are more susceptible than the Gram-positive, while the spores are highly resistant. In addition, the cells in the active phase of growth are much more sensitive that cells in the stationary phase. Moreover, the lethality of the microorganisms during freezing plays a fundamental role in the speed of freezing: the greater the rate of freezing (-18°C in less than 30 min.) the less the cellular damage is.

A greater speed of freezing generally produces less structural damage to the food, due to the formation of smaller crystals (Villani F., 2007). The survival ability of the microorganisms has great interest for the thawing process. It has been observed that the defrosting is intrinsically slower that the freezing and has a trend potentially more harmful. For frozen products the refreezing of defrosted food is not recommend for reasons linked to the consistency, taste, nutritional quality of the product and to the microbiological. Some researchers noted that the products thawed deteriorate more rapidly than the same food stored in a fresh state (Jay *et al*., 2009).

5. Experimental plan

The project was divided in two phases, so two experimental plans were organized:

- *Phase I*: Study of modifications that can occur during freezing storage of buffalo milk and curd;
- *Phase II*: Evaluation of the effects of frozen curd addition in Buffalo Mozzarella cheese

5.1 Experimental plan *Phase I*

In the this first phase of the project, the effects of freezing storage on buffalo milk and intermediates destined to Buffalo PDO Mozzarella cheese-making process were examined through a microbiological, proteomic and lipidomic approach.

Firstly, the effectiveness of the markers identified in scientific literature to identify the presence of frozen intermediates in Buffalo Mozzarella PDO cheese were evaluated.

At the purpose, during Buffalo PDO Mozzarella cheese-making process, samples of milk, curd and cheese were collected. They were analysed in their fresh state and during freezing storage for 9 months. Monthly samples were thawed and analysed, as shown in fig.5.

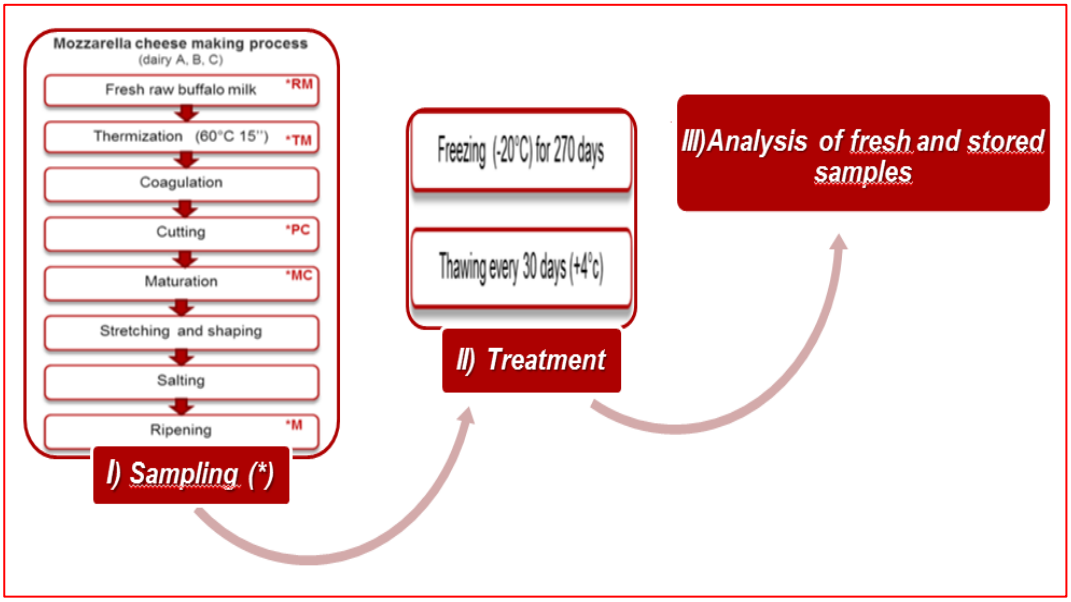


FIGURE 5 EXPERIMENTAL PLAN PHASE I: *RM: RAW MILK ; *TM: THERMIZED MILK ; *PC: PREMATURE CURD (pH 6.2-6.3); *MC: MATURE CURD (pH 5.0-4.8); *M: MOZZARELLA CHEESE

Analytical determinations:

To assess the quality and hygiene parameters of fresh and frozen products, the following determinations were carried out:

- ✓ pH
- ✓ titratable acidity (°SH)
- ✓ water activity
- ✓ somatic cell count (SSC)
- ✓ microbiological analysis
- ✓ chemical composition (fat, protein and moisture content)
- ✓ fat characterization (fat content, fatty acids (FA), mono-diglycerides (MDG), triglycerides (TG) composition, acidity, peroxides value, total polar compounds (TPC) and thermal profile (DSC))
- ✓ furosine content
- ✓ γ4-CN and αS1-CN fragments

5.2 Experimental plan *Phase II*

To evaluate the effects of frozen curd addition in the preparation of Buffalo Mozzarella cheese, the cheese-making processes indicated in the fig. 6 were carried out:

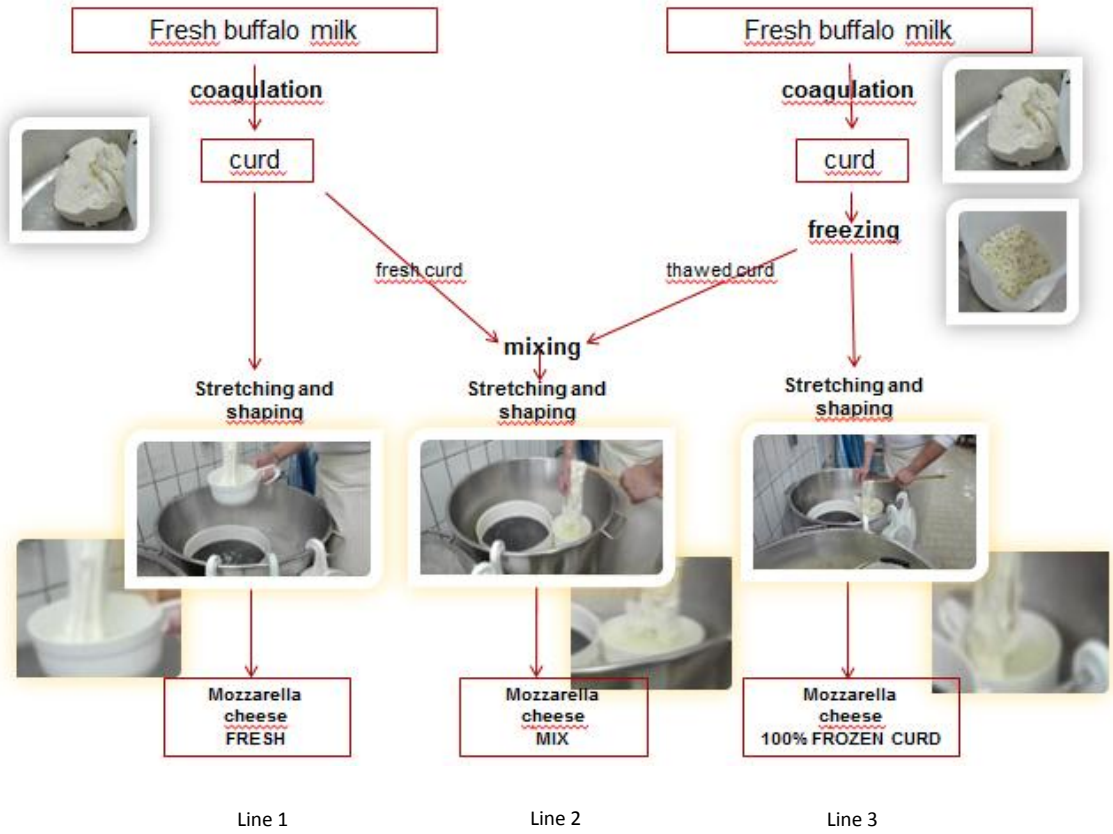


FIGURE 6 EXPERIMENTAL PLAN PHASE II: BUFFALO MOZZARELLA CHEESE-MAKING PROCESS BY FRESH AND FROZEN CURD

In particular, three kind of mozzarella (250g each) were collected:

- Buffalo Mozzarella PDO cheese produced according to the traditional specifications (from fresh milk and fresh curd) (line 1)
- Buffalo Mozzarella cheese by mixing fresh and frozen curd at different percentage (20, 40 and 80%) (line 2)
- Buffalo Mozzarella cheese by 100 % frozen curd (line 3)

Analytical determinations:

The following analysis were carried out to evaluate the effects of frozen curd addition in Buffalo Mozzarella Cheese:

- ✓ basic parameters: pH, moisture, calcium, fat and protein content
- ✓ fat lipolysis and thermal profile (DSC)
- ✓ furosine content
- ✓ structural properties
- ✓ Metabolomic analysis
- ✓ Peptide profile

6. Materials and Methods

Phase I: Study of modifications that can occur during freezing storage of buffalo milk and curd

6.1 Sampling

The samples were collected in three different dairy plants (A, B and C) in province of Caserta, Salerno and Napoli (Campania region, Italy) during Buffalo Mozzarella cheese-making process. All dairy plants were producers of mozzarella with PDO certificate.

Three samplings were performed in each dairy plant.

6.2 Sample treatment

Each sample collected during buffalo mozzarella PDO cheese production was divided into 10 aliquots: the first was used to analyse the fresh product, and the other 9 aliquots were frozen at – 20°C (freezing time: 4 hours) for a total period of 9 months. Monthly, the samples were thawed, and caseins and fat were extracted for the analysis.

To simulate, in the laboratory, the actual condition adopted in dairy plants, a fast freezing was obtained by cutting curd and mozzarella samples to small sizes, nearly 100 g, and freezing them at -20°C. The thawing process was conducted by maintaining the frozen samples at 4°C over-night, and subsequently in a water bath at 40°C. After thawing, they were used for chemical analysis.

The freezing and thawing time-temperature profiles were registered using a data logger (Ebro), which was placed in the core of the sample. Temperature was measured at regular intervals of 30 sec. The thermal profile is reported in fig.7.

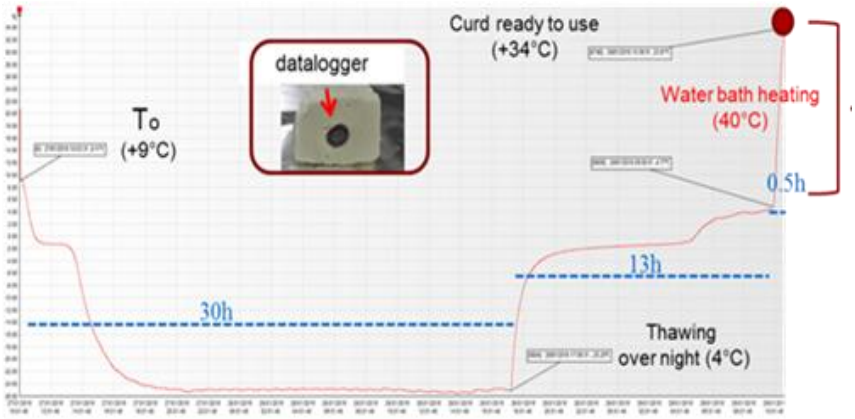


FIGURE 7 TEMPERATURE PROFILE DURING FREEZING AND THAWING PROCESS

6.3 Quality and hygiene parameters

The following analysis were carried out to evaluate the quality of milk used in the cheese production, the quality of mozzarella cheese obtained and to monitor the quality of the food matrices (milk, curd and mozzarella cheese) during the freezing storage:

pH: The pH determination was performed by use of conventional pH meters equipped with a commercially available electrode for liquid samples and a commercially available electrode for solid samples, both with accuracy of ± 0.02

Titratable acidity: The titratable acidity of milk was calculated as the volume of NaOH 0.25 Normal that were necessary to neutralize all the acids present in 100 ml of milk (until pH 8.4), the turning point of the phenolphthalein, indicator for the titration. The degree of acidity was expressed in °SH/100 ml of sample

Water activity: The water activity was measured by The Official Methods ISO 21807:200 at 25°C ± 1°C, with Aqualab 4TE instrument, composed of a probe-container useful for the housing and measurement of the sample and of a portable reader. The equipment is based on the electrical conductivity of the average in regard to a lithium chloride solution reference. The perception is depicted by a sensor that measures the variation of electrical conductivity at a constant temperature in a sealed chamber. The sample was located inside the hermetically sealed probe-container; part of the water inside the sample evaporates, allowing the sensor to detect the degree of free water. This instrument for the measurement of water activity is able to precisely read in about 4 minutes, with an accuracy of ±0.003 Aw at a temperature of 25°C ± 1°C.

The result was conveyed as a percentage of the equilibrium relative humidity (% URE), since aw= p/p0 where p = the value of steam pressure on the surface of the product, therefore % URE = aw . 100 e where p0 = steam pressure on the surface of pure water at the temperature of the sample. (Salvadori del Prato 1998).

SCC: The somatic cells of milk were revealed through a fluorescence microscope Nucleo Counter SCC-109 – ASTORI according to the Internal Method. This method involves adding a DNA intercalation solution (500 µl) to 500 µl of sample. The number of somatic cells was automatically calculated by a software program. The value of this parameter in accordance to Italian law (Regulation 853/2004) for buffalo milk is $\leq$ 400,000* for ml (*rolling geometric average, which was calculated over a period of three months with at least one sample per month from bulk milk, unless otherwise specified by the appropriate authorities).

Fat and protein content: The fat and protein contents in milk were determined by Milko Scan.
The cheese protein content was analysed by the AOAC Official Method 2001.14 (Determination of Nitrogen (Total) in Cheese by Kjeldahl method). This method is applicable to hard, semi-hard and processed cheese with a crude protein content (total nitrogen x 6.38) between 18-36%. Briefly, 1 g of cheese is digested in H2SO4,

using CuSO4 as catalyst, to release nitrogen from protein and retain nitrogen as ammonium salt. Concentrated NaOH is added to release NH3, which is distilled, collected in boric acid solution, and titrated.
The fat content in solid samples was calculated after lipids extraction described in the paragraph 4.6.1

Cheese moisture: Moisture content of cheese was measured by oven drying. An aliquot of 5 g was weighed precisely and heated for 6 h at 130°C. Samples were left to cool in a desiccator until room temperature was reached and then weighed. Moisture content was calculated as the difference between the initial mass and the final mass after drying *100.

Microbiological analysis: The microbiological analysis were carried out in collaboration with the 'Istituto Zooprofilattico Sperimantale del Mezzogiorno' - Department of Foods Inspection, Milk Laboratory (Portici, Campania region, Italy).
The main pathogenic, spoilage and pro-technological bacteria were evaluated in fresh products and during freezing treatment.
For the quantitative analysis, results were expressed in base-10 logarithm and expressed as colony formant unit (CFU) /ml for liquid and CFU/g for solid samples.

- pro-technological bacteria:
  - *Lactic bacteria*
- Spoilage microorganisms:
  - *Total bacterial count*
  - *Coliforms*
  - *Enterobacteriaceae*
  - *Escherichia coli*
  - *Psychrophilic microorganisms*
  - *Pseudomonas spp.*
  - *Coagulase-positive staphylococci*
  - *Yeasts and moulds*
- pathogen bacteria:
  - *Listeria monocytogenes*
  - *Salmonella spp.*
  - *Campylobacter spp.*
  - *Escherichia coli O157:H7*
  - *Bacillus cereus*
  - *Yersinia enterocolitica*

Sample preparation: A representative amount of sample (10 - 25 ml/g) was used. Sterile diluent with a ratio 1:10 was added. Such a dilution, also called first dilution or stock dilution, was made by adding 90 ml of diluent to 10 g/ml of sample. Solid samples were homogenized with a Stomacher, that crushed the sample determining its pulping.

Count of spoilage and pro-technological microorganisms: The microbiological analysis of spoilage and pro-technological microorganisms required a quantitative analysis. For each sample, scale dilutions have been carried out in a Peptone-Saline Solution (PSS) up to a 10^{-7} for the count of spoilage and pro-technological microorganisms, with the exception of the count of moulds and yeasts, where the Peptone Water Broth (PWB) diluent was used. The stock solution was prepared in accordance with the same procedure explained previously. The dilutions have been seeded in triple in the culture mediums. The procedures for microbiological analysis were in accordance with ISO regulations.

Methods used for microbial analysis

Total bacterial count: The method for the plate count at 30°C with the technique of deep pour refers to the UNI EN ISO 4833-1:2013. This is a determination allowing an assessment of the global quality of the product (Quality Index) and provides general information on the times of food conservation. For the solid sample the Plate Agar Count (PCA) medium was used, while for the liquid one the Plate Agar Count (PCA) + skimmed milk (PCM) was required. Plates were incubated at 30°C for 72 hours. All colonies grown were counted, except for yeasts and moulds.
The legal limit in accordance with the Regulation no. 853/2004 of the Parliament and the Council of Ministers of 29/04/2004 expresses the value in germs at 30°C (per ml or g) ≤ 1500,000 (rolling geometric average, calculated over a period of two months, with at least two samplings per month).

Enterobacteriaceae: The method used for the numbering of *Enterobacteriaceae* refers to the ISO 21528-2:2004. The Violet Red Bile Glucose Agar (VBRG) medium was used. Incubation at 37°C for 24 hours. All the colonies presenting a diameter greater than 0.5 mm (red-violet color, with or without red-violet ring) were counted. Then the confirmatory test was carried out using Nutrient Agar and incubation at 37°C for 24 hours. After the incubation, the oxidase reaction test was performed.
According to law 2073/2005 modified in 15/11/2005 the limit is ≤ 10 cfu/ml on five sample units for milk and milk derivatives.

Coliforms: The method used for the count of *coliforms* refers to the ISO 4832:2006. The research of coliforms (quality and hygiene indexes) was carried out using as medium the Agar Crystal Violet Neutral Red Bile Lactose (VRBL). Incubation at 30°C for 24 hours. The confirmatory test was performed in Lactose Brilliant Green Bile Broth tubes containing a Durham bell. The legal limit in accordance with the

Regulation 2073/2005 and Single Supervisory Mechanism (SSM) is 10 cfu/ml on 5 sample units for milk and dairy products.

Escherichia coli: This germs are used as indicator of the hygiene level. The method used refers to the ISO 16649-2:2001. The medium used was the Agar Tryptone-bile-glucuronic (TBX). Incubation at 44°C for 24 hours. The legal limit in accordance with the Regulation 2073/2005 must be between 100 and 1000 cfu/ml or g on of milk and dairy products

Coagulase-positive staphylococci: The method used for the count of *Coagulase-positive staphylococci* refers to the ISO 6888-2:2004. The medium used was the Agar Baird-Parker with Rabbit Plasma Fibrinogen (RPF), allowing the simultaneous count and confirmatory test in a single operation. Incubation at 37°C for 24-48 hours.
Legal limits in accordance with the Regulation 2073/2005 must be between 10 and 100 cfu/ml or g.

Pseudomonas spp.: The method for numbering and researching the *Pseudomonas spp.* refers to the ISO/TS 11059 IDF/RM 225 01-08-2009. The medium used was the Pseudomonas Penicillin Pimaricin Agar base (PPA), with a pimaricin supplement. Incubation at 25°C for 48 hours. A confirmatory test was carried out on Nutrient Agar medium and incubated at 25°C for 24/48 hours and then oxidase test. The colonies considered positive were inoculated in tubes containing the Glucose Agar medium (GA) and incubated at 25°C for 24 hours. The limit based on regional Guidelines is 10^6 cfu / ml or g of milk and dairy products

Moulds and Yeasts: The method refers to the ISO 21527-1:2008. The count was carried out using a Rose Bengal Agar (DRBC Agar).
The limits are comprised between 100 and 1000 cfu/ml or g on milk and milk derivatives (WQA 2011 e FDC 2009)

Psychrotrophs: The method used for counting psychrophilic microorganisms refers to the ISO 17410:2001. The mediums used are the Plate Count Agar (PCA) for the solid sample and the PCA + skimmed milk (PCM) for the liquid sample. Incubation at 6.5°C for 10 days.

Lactic bacteria: The method refers to the ISO 15214:1998. The medium used was MRS (De Man, Rogosa and Sharpe) Agar. Incubation in aerobiosis, at 30°C for 72 hours.

Microbial count technique
The criteria for microbial count employed were represented to the ISO regulation 7218:2007: “General requirements and guidance for microbiological examinations”.
The count was carried out when on at least a plate there were colonies in the measure of 10-300 (total, typical or compliant with the identification criteria).
The N number of microorganisms in the sample was calculated with the arithmetic mean between two successive dilutions using the equation:

$$N = \frac{\Sigma C}{V\ x\ 1{,}1\ x\ d}$$

- *N* = number of microorganisms,
- *ΣC* = sum of the colonies in the plates taken into consideration,
- *V* = volume, expressed in ml, of the inoculum poured on each plate,
- *d* = dilution factor corresponding to the first dilution taken into consideration.

The result was expressed in CFU/g for solid products or CFU/ml for liquid products. In case there of a low count of colonies (<10) or absence thereof, results were reported <10 CFU/g or ml

Research of pathogenic microorganisms
The research of pathogenic microorganisms was carried out as a qualitative assessment, namely 'presence/absence' in a pre-determined quantity of food.

Salmonella spp.: UNI EN ISO 6579:2009. In the regulation CE no. 2073/2005 it is established that Salmonella spp. must be absent in 25 g of product.

Yersinia enterocolitica: ISO 10273:2005. Presence or absence indicated in 10 g or 1 ml of product. In milk and dairy products the *Y. Enterocolitica* must be absent.

Listeria monocytogenes: UNI EN ISO 11290-1:2005. The pathogen must be absent in 25 g or ml of product, in accordance with the Regulation 2073/2005.

Escherichia coli O157:H7: UNI EN ISO 16654:2003. In accordance with Regulation 2073/2005 *E. coli O:157:H7* must be absent in 25 g or ml of product.

Campylobacter spp.: UNI EN ISO 10272-1:2006.

Bacillus cereus: EN-ISO 7932:2005. In accordance with the Regulation (CE) 2073/05 the legal limits are 50 – 500 cfu/g.

6.4 Evaluation of γ4-CN and αS1-CN effectiveness

6.4.1 Casein extraction from milk

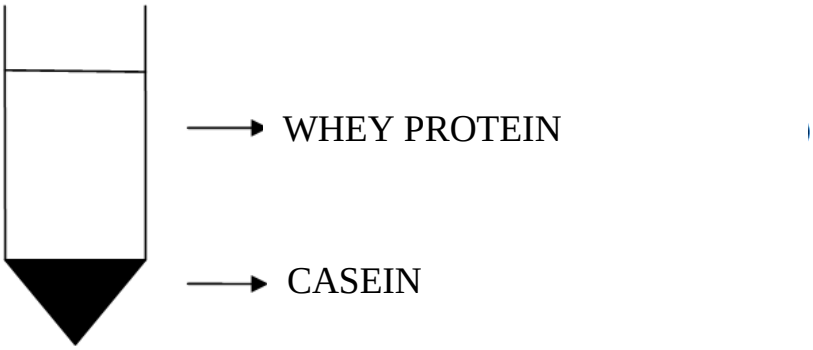
Casein was isolated from fresh and frozen samples by isoelectric precipitation at pH 4.6 by addition of acetic acid. The preparation was performed according to the Ashaffenburg & Drewry method.

Reagents:

- ACETIC ACID 10%
- SODIUM ACETATE 1 N
- Washing solution (50 ml glacial acetic acid 10% plus 50 ml of sodium acetate 1N diluted to 1 L with a final pH of 4.6)
- DICHLOROMETHANE
- ACETONE

Procedure:

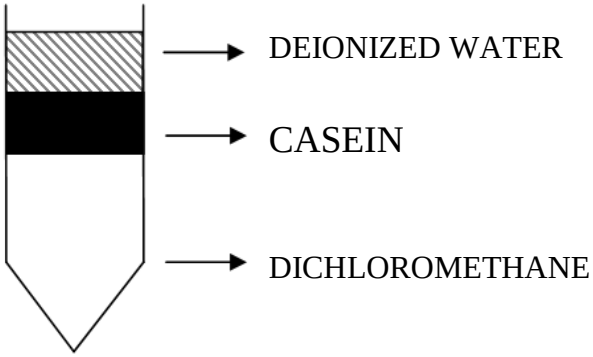
- 50ml of milk centrifuged at 4000 rpm for 10 minutes
- refrigerated for 5-10 minutes to remove easily the fat
- the remaining samples was divide into two test tubes and 20ml of milk to 20 ml of deionized H_2O were added in each test tube
- 5 ml of 10% of acetic acid were added and shaked, then 5 ml of sodium acetate 1N were added
- centrifugation at 4000 rpm for 10 minutes to obtain the casein separation:



- The serum proteins were removed and caseins subjected to further washing to eliminate completely serum proteins.

Casein washing:

- 20 ml of the washing solution (50 ml acetic acid 10% plus 50 ml sodium acetate 1N diluted to one liter of deionized water (pH 4.6))were added and the caseins were crushed with a spatula
- centrifugation at 4000 rpm for 10 minutes
- washing solution was discarded and washing was repeated twice
- 30 ml of deionized water were added and mix with Turrax to break up the casein on the bottom of the tube
- Checking that the pH was 4.6
- 20 ml of dichloromethane were added
- centrifugation at 4000 rpm for 10 minutes to obtain:



- Deionized water and the dichloromethane were removed taking care not to break or release casein from the tube
- proceeding with dichloromethane repeated until both phases of extraction became clear, approximately 2 or 3 times.
- To the purified caseins 50 ml of acetone were added
- centrifugation at 4000 rpm for 10 minutes
- acetone removed from the tube and protein extract mashed to form a thin film on a surface and left overnight in a fume cupboard until obtain a fine powder. Samples were sealed and kept in a freezer at -20°C.

6.4.2 Casein extraction from curd and cheese

Casein isolation was carried out according to the method described in the EC REGULATION 273/2008 ALL 9 par. 6.1

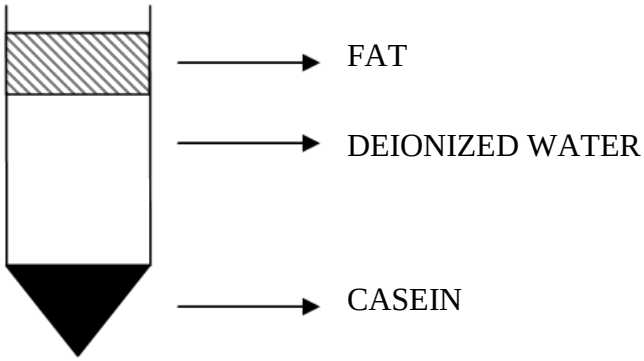
Reagents:

- Acetic acid 25%
- Dichloromethane
- Acetone

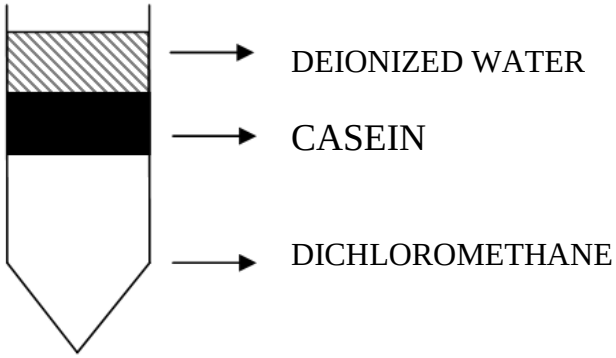
Procedure:

- 5 g of samples were weighted in a test tube
- 30 ml of deionized water were added and homogenized with a Turrax (8000-10000 rpm)

- 20 ml of deionized water were added
- checking that pH was 4.6
- centrifugation at 4000 rpm for 10 minutes to obtain:



- fat and the serum were removed
- 30 ml of deionized water were added and mix with Turrax to break up the casein on the bottom of the tube
- Checking that the pH was 4.6
- 20 ml of dichloromethane were added
- centrifugation at 4000 rpm for 10 minutes to obtain:



The washings with dichloromethane and acetone were performed as for milk samples until to obtain a purified dried caseins powder.

6.4.3 γ4-CN determination

SDS-PAGE (Sodium Dodecyl Sulphate - PolyAcrylamide Gel Electrophoresis) analysis was performed for the identification of. The analysis was carried out according to

the Official Method N. L74/25 of 20.03.1992, and a subsequent densitometric analysis (GS-800 Bio-Rad).

Preparation of samples

- 10 mg of dried casein were weighted and the following reagents were added:
- 10 ml of 9M urea prepared by dissolving 54.05 g of urea in 100 ml of deionized water
- 10 µl of β-mercaptoethanol ready to use Bio-Rad
- Vortex to allow the solubilization of casein
- Then, addition of:
- 1 ml of Fullington solution (750 mg TRIS, 2 g of SDS and 5 ml of β-mercaptoethanol at pH 6.8 in 100 ml)
- sucrose
- 10 µl of phenol blue bromine (10 x Blue Juice Invidrogen)

The samples were subjected to denaturation by boiling at 100° C for 10 minutes and subsequently cooled prior to loading into the gel.

Preparation of gel and running buffer

RUNNING GEL (12%)

- Water (16.5ml)
- 30% Bis-Acrylamide Acrylamide (20.0 ml)
- 1.5 M Tris pH 8.8 (45,43 g tris in 250 ml of water, pH 8.8) (12.5 ml)
- 10% SDS (500 ul)
- 10% ammonium persulfate (APS) (500 ul)
- TEMED (tetramethylethylenediamine) (20 ul)

STACKING GEL (5%)

- Water (6.8 ml)
- 30% Acrylamide Bis-Acrylamide (1.7 ml)
- 1 M Tris pH 6,8 (1.25 ml)
- 10% SDS (100 µl)
- 10% APS (100 µl
- Temed (20 µl)

RUNNING BUFFER

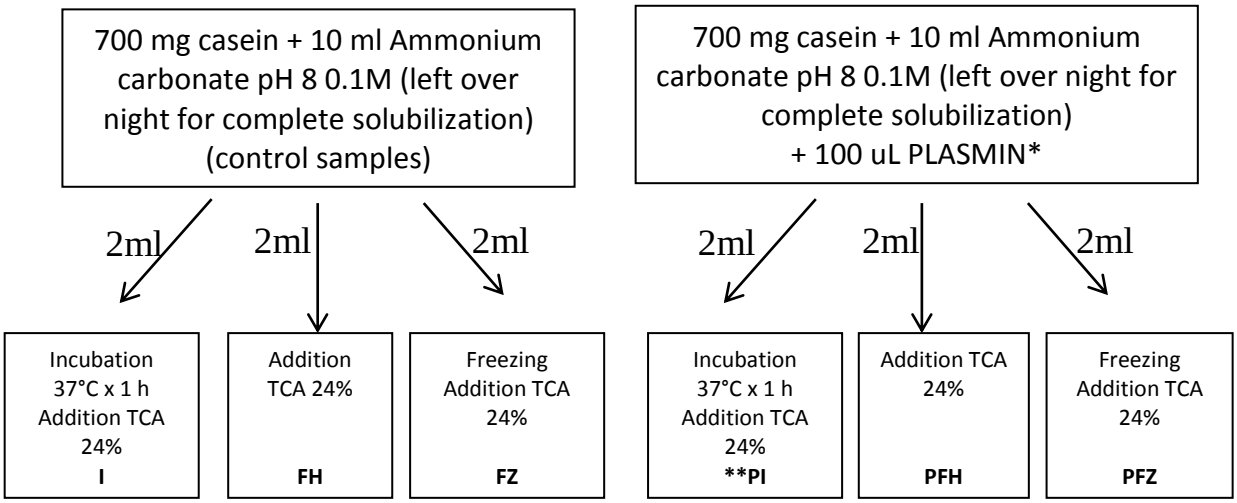
- Tris (15.1 g)
- Glycine (72 g)
- SDS (5 g)
- All dissolved in 5 liters of deionized water and adjusted to pH of 8.6.

A volume of 20 µl of sample was loaded into the gel

6.4.4 Plasminolysis

For plasminolysis analysis, caseins were extracted by fresh buffalo milk as indicated above.

Then, the scheme indicated was followed:



All samples were centrifuged at 4000g x 10 min and precipitated proteins were recovered.
For SDS-PAGE analysis, 2ml UREA 9M and 20 µl 2-mercaptoethanol were added in each sample. Samples were vortexed until complete dissolution of caseins.
An aliquot of 1ml was transferred in an eppendorf tube and then 1ml Fullington solution, sucrose and10µl blue phenol bromine were added.
Finally samples were vortexed and subjected to the denaturation treatment (100°C for 10 min).

*Plasmin 5 U from bovine plasma was used.
** PI was also indicated HP in the gels where the comparison with hydrolyzed milk was shown.

6.4.5 αS1-CN determination

UREA PAGE was performed to evaluate αS1-CN fragments.

Proteins were separated on the basis of their negative charge. A gel with a basic pH of 8.6 was used.

Preparation of the samples

- 10 mg of casein were weighted in a 1.5 ml Eppendorf.
- 1 ml of 9 M urea and 10 ml of β –mercaptoethanol were added.
- After shaking, 500 µl were poured into a 1.5 ml Eppendorf.
- 500 µl of glycerol at 75 % (P/V) were added
- 10 µl of Blue phenol bromine (BB) were added and solution mixed

RUNNING GEL

- UREA 9M (15.54 g)
- Tris-HCl 1.5 M pH 8.8 (7.5 ml)
- Distilled Water (10.5 ml)
- Acrylamide-Bis-Acrylamide 30% (10.5 ml)
- TEMED (20ul)
- ammonium persulfate (APS) 22% (200 ul)

STACKING GEL

- UREA 9M (2.2 g)
- Tris-HCl 0.5 M pH 6.8 (2 ml)
- 30% Bis-Acrylamide Acrylamide (1 ml)
- Glycerin 75 % (w/v) (1 ml)
- Distilled Water (4 ml)
- TEMED (4µl)
- Ammonium persulfate (APS) 22% (30µl)
- Load 10 µl of the sample into the gel.

RUNNING BUFFER:

- Glycine (72 g)
- Tris (15.1 g)

Diluted to a volume of 5 l with deionized water. Final pH of 8.6.

A volume of 20 ul of sample was loaded into the gel

Since αs1 caseins are mainly formed by the action of chymosin (exogenous enzyme added in the cheese-making process), a hydrolyzed sample whit the enzyme was prepared to have a positive control in the gels interpretation.

6.5 Furosine determination

To analyse the furosine content in fresh and frozen curd, an acid hydrolysis of samples was carried out. A volume of 6 ml HCl 10,6 N was added to 300mg of sample.
Then, the sample was held under nitrogen for 2 min and heated at 110°C for 23 h.
After hydrolysis, samples were filtered on paper and 2 ml of obtained solution were used for Kjeldahl analysis to determine protein content.
Filtered sample was purified with solid phase extraction method (Discovery DSC-18, Supelco, USA-500mg). Briefly, 5 ml of methanol and 10 ml of water were used to activate the phase. Then, 0.5 ml of filtrate were loaded into the phase and eluted sample was discarded. Subsequently, 3 ml HCl 3 N were added and purified sample was collected and analysed by HPLC.
The quantification was performed with an external standard (Furosine Dihydrochloride polypeptide group 10 mg (Strasbourg, France)). A concentration of 0.977362 nmol/ml in HCl 3N was injected.
HPLC analysis: HPLC Agilent 1100 Series, volume injection 20 µl, DAD detector, C8 column (Alltech "Furosinee dedicated" (250 mm x 4,6 mm), Alltech-Italia, Sedriano-Milano). Furosine was detected at 280 nm.

Chromatographic conditions:
Eluent A: 0.4% acetic acid in water (v/v)
Eluent B: 0.3% potassium chloride in eluent A (p/v)
Flow rate: 1.2 ml/min

Gradient elution program:

time (min)	% A	% B
0	100	0
13.5	100	0
20.5	50	50
22.0	50	50
23.0	100	0
32.0	100	0

The adopted method is described in the Gazzetta ufficiale DM n.69,1994.
Furosine amount was expressed as mg/100 g of proteins after calculation with the following formula:

$$\frac{Ac \times Cs \times 1 \times 6 \times 254 \times 8}{As \quad v \quad 0.95 \quad 10 \quad m \times 4}$$

Ac = peak area of furosine in the sample
Cs = furosin amount of injected standard (picomole)
As = peak area of furosine standard
v = volum of injection (µl)
6 = dilution factor after solid extraction
0.95 = recovery factor of furosine in solid phase extraction

254 = furosine molecular weight
m = protein content in 2 ml of hydrolyzed sample, express in mg

6.6 Lipids analysis

6.6.1 Fat extraction

Lipids were extracted according to the Schimith-Bondzynsky-Ratzlaff method (Official Method of Cheese Analysis (D.M.1986)) with some modifications. Briefly, liquid samples were weighted and added with ethanol. Then a solution ethyl ether/heptane 2:1 (v/v) was added to extract the fat (for 3 times). For solid samples, an acid hydrolysis with chloric acid at 37% was executed to recover fat and then the same procedure described for liquid samples was performed. The yield of extraction was calculated by gravimetric method:
FAT % (w/w) = grams of fat extracted / grams of initial sample weighted * 100

6.6.2 Fatty acid analysis

The fatty acid profile was analysed by using gas chromatography coupled with an FID detector (Agilent Technologies 6850 Series II). The GC was equipped with a capillary column 50% Cyanopropyl Methyl Silicon, 100 m 0.25 mm ID 0.20µm. The oven program temperature was: 140°C x 5 min, increase 4°C/min until reaching 175°C for 20 min, then increase 3°C/min until reaching 240°C held for 1 min. The injector program temperature was: 60°C x 0.1 min, with an increase of 500°C/min until reaching 260°C held for 5 min. Helium was used as the carrier gas (flow rate 1.8 ml/min). The detector temperature was set at 260°C.
A solution of fat 5% in hexane was injected, after trans-esterification with 2N KOH. Identification was performed with a 37 component FAME mix standard from Supelco, St.Louis, Mo, USA.

6.6.3 Triglycerides analysis

Triglycerides analysis was performed by gas chromatography (DANI GC1000) coupled with an FID detector. The GC was equipped with a capillary column 65%phenyl 35%methyl-polisiloxane, 30 m x 0.25 mm ID x 0.1µm.
The oven program temperature was: 250°x 2 min, increase 6°C/min until reaching 365°C for 10 min. The injector program temperature was: 60°C x 30 sec, with an increase of 500°C/min until reaching 300°C held for 7 min
Helium was used as the carrier gas (flow rate 1.5 ml/min). The detector temperature was set at 370°C.
A solution of fat 4% in hexane was injected

6.6.4 Lipolysis and oxidation

Free fatty acids (FFA), peroxides value (PV), total polar compounds (TPC) and mono-diglycerides (MDG) were analysed to evaluate the alteration of fat during the freezing storage.

FFA analysis: To quantify free fatty acids a titration method was used. 1g of fat was weighted and 16 ml of ethanol/ethyl-ether (1:2) were added. NaOH 0.005N was used to neutralise sample acidity and phenolphthalein was used as indicator. Results are expressed ad % of oleic acid

PV analysis: Peroxides value was determined using a modified method of Shantha and Decker (1994). Briefly, 500 mg of extracted lipids were put in a 20 ml flask and mixed with 10 mL chloroform/methanol (7:3, v/v). then, 0.05 ml of ammonium thiocyanate (30g/100 in water) and 0.05 ml of iron chloride solution (II) were added and vortexed. Then chloroform/methanol (7:3, v/v) solution was added until 20 ml. After 20 min, the absorbance was measured at 500 nm using a double beam UV-VIS spectrophotometer. PO was calculated using a Fe (III) standard calibration curve with a concentration range of 0.1-0.5 ppm. Results were expressed as meq O2/kg.

TPC: The total polar compounds were analysed by a procedure described in Caboni et al., 1996 with some modifications. The fat extracted from the fresh and frozen samples was separated using the SPE column (Discovery SPE DSC-Si Silica Tube 52658- U - 20 ml). The sample of fat was dissolved in chloroform and betulin was added as internal standard.
Triglycerides (TG) were separated with hexane/ethyl ether solution (15: 1)
Mono-diglycerides (MDG) were separated with hexane/ethyl ether solution (1: 1)
Phospholipides (P) were separated by using methanol
Every fraction was dried under vacuum at 40°C and the results expressed in % of total fat.

MDG analysis: To avoid the interference of triglycerides (TG) with mono and di-glycerides (MDG), the sample of MDG fraction separated by the previously described procedure for TPC analysis was used.
The MDG fraction was analysed by gas chromatography coupled with mass spectrometry detector (Agilent Technologies 5977E MSD, Agilent Technologies 7820A GC system, Autosampler), using a capillary column DB-5, 25m x 0.247 mm I.D., film thickness 0.25 µm. The oven program temperature was: 80 °C held for 0.5 min. Increase of 10 °C/min until 340 °C held for 30 min. Mass range was 40-900. Inlet was set at 320 °C. Betulin was used as an internal standard. The samples were analysed as trimethylsilyl (TMS) derivatives. As reported by other authors, NIST and Wiley libraries do not provide the mass spectra for all products of glycerol esterification. In particular, there is no single mass spectrum of diglycerides with different substitutes (Isidorov et al., 2007).
For the quantification, we assumed that all monoglycerides had an identical response factor as monoolein, and all diglycerides had an identical response factor as 1,2 diolein, which were used as external standards.

6.6.5 Thermal profile analysis

The thermal profile of fat extracted from fresh and frozen curd was evaluated.

The analytical procedure described in Fatouth et al., 2007 was followed. A model 7 Perkin Elmer differential scanning calorimeter (DSC) (Perkin Elmer, Norwalk, CT) was used to study the thermal profile of fresh curd, frozen curd, fresh mozzarella cheese and mozzarella produced from use of frozen curd. The DSC was calibrated using indium (m.p. 156.60 1C, DHf 28.45 J/g) and Gallium (m.p. 29.78 1C, DHf 80.09 J/g). Samples (9–10 mg hermetically sealed in aluminum pans) were heated to 80 °C and held at this temperature for 5 min, and then cooled to 50 °C at a rate of 10 1C/min to obtain the crystallization curves. After 15 min holding at 50 °C, melting curves were generated by heating the samples again to 80 °C at a rate of 10 °C/min.

Phase II: Evaluation of the effects of frozen curd addition in Buffalo Mozzarella cheese

6.7 Sampling

The samples were collected in three different dairy plants in province of Caserta, Salerno and Napoli (Campania region, Italy). All dairy plants were producers of mozzarella with PDO certificate. Three samplings were performed in each dairy plant.

6.8 Sample treatment

For the preparation of Mozzarella Cheese from fresh and frozen intermediates, fresh curd was stored in 12-15kg polyethylene bags at freezing temperature (-20°C) for 6 months. Then, at the moment of sampling, it was thawed and used as frozen curd. All samples underwent the same production procedures and technological processes and were delivered to the laboratory few hours after preparation. Each sample was contained inside an individual package, completely immersed in mozzarella whey.

6.9 Basic parameters, fat oxidation, fat thermal profile and furosine content

pH, moisture, fat and protein content: The basic parameters of mozzarella cheese obtained by fresh and frozen curd the fat oxidation and furosine content were evaluated as previously described in *Phase I*.

Calcium determination: For determination of total calcium, 5 g of sample were heated at 525°C for 12h. Then, ashes were dissolved in water and sulphuric acid. Then, the sample was titrated with EDTA M/400.
For soluble calcium, the procedure reported in Metzger et al. (2001) was followed. Briefly, 5g of sample were added with water, homogenized and centrifuged. Filtered sample was titrated with EDTA 0,01M.

DSC: The DSC analysis was carried out on fat and on milk fat globule membrane (MFGM) extracted from fresh mozzarella cheese and mozzarella cheese obtained from frozen curd.
Changes in the microenvironment of membrane lipids can be detected by studying the thermotropic properties of the membrane lipids.

Isolation of MFGM from Cheese Whey: The MFGM in cheese whey can be selectively precipitated at pH 4.2 and 30-35 °C when the conductivity of the whey is decreased to below a critical level (Damodaran, 2011).
The MFGM was isolated from Mozzarella cheese whey (fresh, 20% and 100% frozen curd) using a modified method described in Zhu and Damodaran 2011. Briefly,

cheese whey was first filtered on paper, then it was centrifuged at 6320 rpm x 7 min in 10 kDa molecular weight cutoff Millipore Centricon tubes. Subsequently, the pH and the conductivity of the retentate were measured and reported in tab.3.

TABLE 3 PH AND CONDUCTIVITY VALUES OF WHEY EXTRACTED FROM FRESH MOZZARELLA AND MOZZARELLA OBTAINED FROM FROZEN CURD

Sample of whey from	pH	Conductivity (uS/cm)
Fresh mozzarella cheese	6.40	800.01
Mozzarella cheese from 20% frozen curd	6.35	0.04
Mozzarella cheese from 100% frozen curd	6.26	390.13

The stability of MFGM in cheese whey against precipitation is due to the presence of mineral salts, especially Ca2+ and Mg2+, which alter the electrostatic properties of MFGM and prevent it from flocculation/precipitation near its isoelectric point. Therefore the removal of mineral salts from cheese whey may facilitate precipitation of MFGM under certain pH and temperature conditions. Zinc acetate 0.025M was used to induce precipitation of MFGM. The pH of the retentate was adjusted to 4.20 with HCl 1 M and incubated for 30 min at 35°C. The retentate was then centrifuged at 3140 rpm to recover the solid part , which is the MFGM material.

6.10 Structural properties of Buffalo Mozzarella Cheese from frozen curd

Structural properties of Buffalo Mozzarella cheese obtained from fresh and frozen curd were analysed by texture analysis, X-Ray and Nuclear Magnetic Resonance (NMR).

Texture analysis was performed by TA.XT.PLUS (Stable Micro Systems) equipped with a blade set with knife (fig.8). The analysis was conducted on slices (4x1x1.5 cm) removed from the core of Mozzarella cheese samples and slices sampled in the lateral side of Mozzarella samples. The probe was moved at a constant speed (0.50 mm/s) until the complete cutting of the piece. No gap between probe and sample was set at the starting point of analysis. The force values obtained during the test were recorded and hardness was expressed as the total positive area under the curve of cutting test.

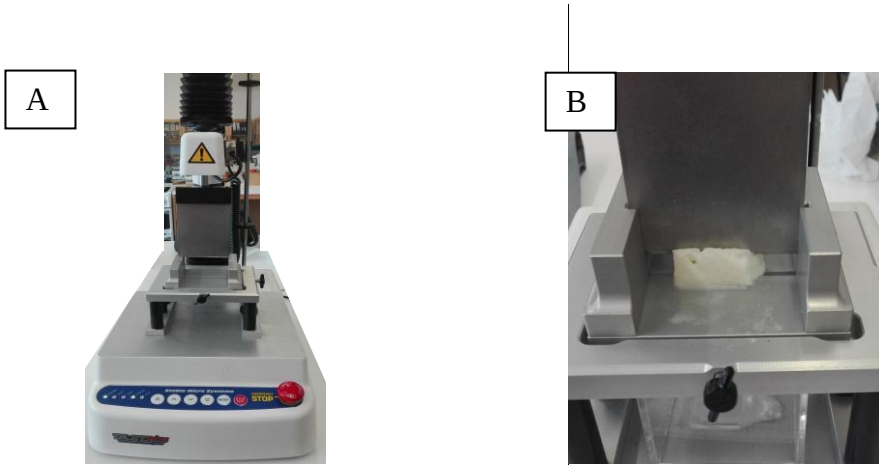


FIGURE 8 CUTTING TEST OF MOZZARELLA CHEESE SAMPLES: INSTRUMENT EQUIPMENT (A) AND SAMPLE TEST(B)

XRay-microtomography: Mozzarella cheese was analysed by XRay-microtomography (Xray mCT) to study the internal structure of the cheese as affected by using frozen curd in cheese-making process. Samples were scanned using a Nikon HMX 225 X-ray microtomography system.

Mechanism of X Ray action: The internal structure is evaluated by means of a X-ray source and a detector able to take information from a projected slice (Kotwaliwale et al., 2014). The principle is based on image contrast that is produced by variations in the X-ray attenuation that includes absorption and scattering (Lim & Barigou, 2004). When an X-ray beam passes through a sample it is attenuated. The differences in attenuation are attributable to density and compositional differences within a sample. Thus the transmission level of the X-ray is determined by the mass as well as the absorption coefficient of a sample. During image acquisition an X-ray beam, which is collimated, is directed toward a sample, the detector measures the remnant attenuated radiation and the response is transferred to a computer. This radiation type has the ability to penetrate a sample in varying degrees (Cnudde & Boone, 2013).
Before scanning, instrumental conditions such as beam energy and current, sample-to-detector distance and exposure time, are optimized. During scanning the sample is rotated on a translation stage while illuminated with X-rays (Baker et al., 2012). The X-rays pass through the object in many different directions and along different pathways to create an image illustrating variation in density at numerous points in a 2D slice (Lim & Barigou, 2004). As the sample rotates, a series of 2D radiographs or projection images are acquired (Frisullo et al., 2009). The detector records the object that is transversed by the X-ray beam.

Image analysis: Data from numerous X-ray radiographs are processed with a computer to reconstruct a 3D volume. Tomograms, which are the 3D representation of a sample's internal structure and composition, can be extracted from these 3D volumes. This image is comprised of volume elements (voxels) that represent the X-ray absorption at a specific point (Landis & Keane, 2010). The images can be presented as virtual slices at various depths and in various directions or the sample can be viewed as a whole. Dedicated software packages enable

manipulation and analysis of the data as well as reconstruction of cross-sections along any orientation. Image contrast is due to differences in X-ray absorption and is caused by density and compositional variation in the sample. It is the association between X-ray absorption and object density that enables the 3D internal structure to be visualized (Landis & Keane, 2010). Thus, the images obtained could be considered a map of the X-ray spatial distribution, where the brighter regions correspond with a higher density (Frisullo et al., 2009).
Images were analysed by VG Studio Max 3.0 program.

Sample preparation: Sample were dried before analysis because of moisture interference. The analysis were carried out on slices (4x1x1) and on whole samples of mozzarella cheese previously freezy-dried.

Experimental condition: The experimental conditions were optimized to allow high quality radiographic slices (free of ring artifacts and beam hardening) and based on the compromise of enhancing both the contrast and resolution of the images using the shortest scanning time (2.5 hours).

Low-resolution NMR: Nuclear magnetic resonance was used to assess relaxation times of mozzarella cheese produced by fresh and frozen curd. The Minispec TD NMR MQ 20 Bruker instrument was used for the analysis. Setting parameters: 16 scan, gain 65%, τ 0.3 ms and CPMG sequence. CPMG curves were analysed by R software.

Sample preparation: Each Mozzarella sample was divided into four parts. From two of those slices, cylindrical shaped pieces (height 1 cm) were cut and put in NMR tubes.

6.11 H-NMR metabolomic analysis to assess traceability of frozen curd in buffalo mozzarella PDO cheese

Each sample of mozzarella (5g) was extracted with 2 mL H_2O and centrifuged for 15' a 4 °C - 14000 rpm, then 1 ml of extracted phase was recovered.
After, 100 uL of D_2O and 10 uL of DSS 100mM were added for the NMR analysis. Spectra were recorded at 298 K with an AVANCE spectrometer (Bruker BioSpin, Karlsruhe, Germany) operating at a frequency of 600.13 MHz, equipped with an autosampler with 60 holders. NOESY and CPMG spectra were obtained.
A total of 54 samples were analysed. Samples were tested at day 2, 3 and 4 after manufacturing (t2, t3 and t4).

Acquisition parameters for NOESY spectra:

- Set the correct solvent eda|solvent (H2O + D20)
- eda|Pulprog = noesygppr1d.comp
- eda|TD = 32768 (Number of time domain data point)
- eda|SW = 11.9878ppm (Spectral width)
- eda|NS = 4 (Number of scans per block)

- eda|TD0 = 64 (Number blocks)
- eda|DS = 4 (Dummy scans)
- eda|AQ = 2.28 s (Acquisition time ACQ)
- Total acquisition time ~ 44 min 43 s for 256 scans

Acquisition parameters for CPMG spectra:

- Set the correct solvent eda|solvent (H2O + D20)
- eda|Pulprog = cpmgpr1d (presaturation CPMG version)
- eda|TD = 32768 (Number of time domain data point)
- eda|SW = 11.9705 ppm (Spectral width)
- eda|NS = 8 (Number of scans per block)
- eda|TD0 = 32 (Number blocks)
- eda|DS = 16 (Dummy scans)
- eda|AQ = 2.28 s (Acquisition time ACQ)
- eda|D "array" [D1] = 4.0 s (Relaxation delay - RD)
- eda|D "array" [D20] = 30 µs (spin echo delay *t*)
- eda|L "array" [L4] = 128 (loop counter)
- Total acquisition time ~ 28 min 59 s for 256 scans

6.12 Protein and peptide profiles of Mozzarella cheese soluble fraction

Protein extraction from Mozzarella cheese: Protein and peptides from the soluble fractions of cheese were prepared from a 5g entire piece of Mozzarella using 10 ml of a 5% formic acid buffer. After 30 sec, sample was centrifuged and the liquid phase collected.
For limited protein experiments, a 5g sample was incubated in the same buffer and with the addition of pepsin (Sigma, Italy) in a w/w ratio 1.200 to simulate gastrointestinal digestion. Incubation was carried out at 37°C and aliquots of 1ml samples were taken at fixed times from 0 to 150 min.
Sample were then analysed by MALDI TOF MS

MALDI-TOF-MS protein identification: MALDI-TOF mass spectra of proteins and peptides were acquired on a Voyager DE-Pro spectrometer (PerSeptive BioSystems, Framingham, MA) equipped with a N2 laser (λ = 337 nm), using α-cyano-4-hydroxy-cinnamic acid as the matrix (10 mg mL-1 in 50% acetonitrile, v/v, containing 0.1% TFA). Mass spectra were acquired in the reflector positive ion mode using the Delay Extraction (DE) technology. The accelerating voltage was 20 kV. External mass calibration was performed with a commercial mixture of standard peptides (PerSeptive Biosystems, Framingham, MA). A resolution of >8.000 was calculated in the working mass range. Raw data were analyzed using the Data Explorer 4.0 software furnished with the spectrometer. Approximately 200 laser shots were acquired for each segment. Fragmented ions were refocused onto the final detector by stepping down the voltage applied to the reflector. Finally, the individual segments were stitched together using the software purchased with the instrument. Peptide mass fingerprinting (PMF)-based identifications were carried out interrogating the not redundant National Center for Biotechnology Information

(nrNCBI) and Swiss-Prot/TrEMBL databases with Mascot (Matrix Science, London, UK) and Protein Prospector MS-FIT (http://prospector.ucsf.edu/) search engines. Mass tolerance of 0.3 Da, variable pyro-glutamic acid formation at N-terminal Gln and possible methionine oxidation were set as search parameters. Searches were taxonomically restricted to Bubalus bubalis and Bos taurus, whose genome has been completely sequenced.

6.13 Statistical analysis

All experiments were performed in triplicate and results are the average values of three determinations. The data were analysed by ANOVA XL-STAT.
H-NMR spectra were examined by PCA (Principal Component Analysis) using TopSpin 3.5pl6 and analysed with R 3.3.2 version.

7. Results and discussion

Phase I: Study of modifications that can occur during freezing storage of buffalo milk and curd

7.1 Quality and hygiene parameters

pH and titratable acidity

The fresh milk, both raw and thermized, showed pH values between 6.61 and 6.78. In fresh mature curd and fresh mozzarella cheese values between 5.94 to 5.52 were detected. After freezing storage, the pH showed a steady trend without significant variations. Only premature curd showed a decreasing pH value during the first month of freezing. This trend can be attributed to a high content of sweet whey, still subjected to lactic bacteria fermentation (Guimãraes *et al.* 2010).

Titratable acidity of fresh milk showed values between 8.9 and 9.0 SH and no significant changes were observed during freezing.

Water activity

Water activity in the fresh premature curd , mature curd and mozzarella cheese showed similar values between 1.000 and 0.994. After freezing storage, a general reduction of the water activity values was observed. High values of water activity allow proliferation of microorganisms. A reduction of these value means less water for the metabolism of microorganisms (Jay et al., 2009).

SCC

The somatic cells are made up of leukocytes, lymphocytes and flaking cells. These compounds have the role of body defense. They can indicate the status of the animal's health. In fact, the number of somatic cells may undergo to drastic variations in the case of udder mastitis (Salvatore del Prato 1998).

Pasteurization can destroy the somatic cells but it does not stop their enzymatic action which is neither stopped by cold temperatures. The value of this parameter should be as low as possible for the milk destined to make cheese.

The fresh buffalo milk samples used in this study showed a SCC value under the limits indicated by Reg. EC 853/2004 (maximum value 400,000 cells/ml). in fact, the detected values ranged from 167,000 to 281,000 cell/ml.

Microbiological analysis

Microbial analysis were performed to evaluate the status of the fresh milk used for mozzarella production, the quality of intermediates and of the final cheese obtained. The contamination levels were then assessed during the freezing treatment.

The freezing is one of the methods used to prolong the storage of the products. The risk of microbial proliferation is represented by thawing, where the number of microorganisms can rapidly increase, and the development of pathogens dangerous for the consumer's health might occur.

Protechnological, spoilage and pathogen microorganisms were evaluated.

Among protechnological bacteria, *Lactic bacteria* (LB) showed values of 6.6 and 6.5 log CFU/ml in raw and thermized milk respectively.
In normal conditions, buffalo milk contains some strains of Lactobacillus in higher concentrations respect to bovine milk. By metabolic activity of these microorganisms, the flavor and typical aroma of buffalo mozzarella cheese originates.
Values of 6.8-6.9 log CFU/g were found in premature and mature curd respectively, while 4.0 log CFU/g were found in mozzarella cheese. A decrease of LB was observed after freezing storage. In particular, at the end of freezing treatment, reductions of nearly 13% in raw milk, 21% in thermized milk, 30% in premature curd and 47-48% in mature curd and mozzarella cheese were observed.

As regards spoilage microorganisms, Regulation n°853/2004 establishes a maximum legal limit for the total bacterial count (TBC) of 1500,000 CFU/ ml or g at 30°C.
In fresh buffalo milk a TBC value ranging between 6.3 and 4.5 log CFU/ml g was detected (under the legal limit).
In mozzarella cheese TBC was less than 4 log CFU/g and this value remained unchanged with the freezing treatment, ensuring healthy of the product. For the curd samples, a decrease of TBC values was observed during frozen storage.

Coagulase-positive Staphylococci were present in concentration of 3.2 and 2.4 log CFU/ml in fresh raw and thermized milk respectively. Amounts of 1.5 and 1.3 log CFU/g in premature and mature curds were observed. A significant decrease was obtained in thermized milk.

Enterobacteriaceae were present in concentration between 5.4 and 4.6 log CFU/ml or g in fresh milk and curd, while in fresh mozzarella cheese a lower amount of 1.7 log CFU/g was detected. During freezing storage, a decrease of *Enterobacteriaceae* was observed: 80% in mature curd and from 40 to 60% in milk and mozzarella cheese.

Coliforms were found in concentration of 5-5.5 log CFU/ml in thermized and raw milk, while 4-4.7 log CFU/g were detected in mature and premature curds. Mozzarella cheese showed the lowest concentration, with value of 1.7 log CFU/g. During freezing, *Coliforms* decreased in all samples analyzed.

Similar results were obtained by Tejada et al. (2002) where a decrease of *Enterobacteriaceae, Coliforms and Staphylococci* counts were observed during the frozen storage of sheep milk for a period of 9 months.

Escherichia coli β-glucuronidase positive decreased of 53% in raw milk, 66% in mature curd and 70% in thermized milk and in premature curd.

Pseudomonas was present in initial values of 7.0-3.8 logCFU/ml in raw and thermized milks. In premature and mature curds a concentration of 2.7-2.4 logCFU/g was detected.

Psychrotrophs, in the fresh state, were found in concentrations of 7.0 in raw milk, 6.5 logCFU/ml in thermized milk, 5.1 logCFU/g in premature curd, 3.4 logCFU/g in mature curd and 2.6 logCFU/g in mozzarella cheese.

Yeasts ranged from 2.4 to 3.8 logCFU/ml-g while moulds from 1.2 to 3.0 log/ml or g. During freezing, moulds and yeasts decreased in all samples.

No positive results were obtained for pathogenic microorganisms.

Therefore, the fresh raw material showed quality and hygiene parameters within the limits provided by the current legislation.

Milk chemical composition

The chemical parameters of milk sampled in dairy A, B and C, used for Mozzarella cheese production, are reported in tab.4

TABLE 4 FAT,PROTEIN AND LACTOSE CONTENT (%) IN FRESH BUFFALO MILK

	Dairy		
	A	**B**	**C**
Fat (%)	7.2 ± 0.1	8.0 ± 0.2	8.5 ± 0.1
Protein (%)	4.3 ± 0.1	4.5 ± 0.1	4.4 ± 0.2
Lactose (%)	4.7 ± 0.2	4.5±.01	4.6 ± 0.1

The fat and protein contents were within the limits established by the PDO Mozzarella cheese specification: minimum fat content 7.2 % and minimum protein content 4.2 %.

As expected, the PDO Mozzarella cheeses produced in the three dairy plants showed a chemical composition within the standard established by PDO specifications: minimum fat content/dry matter of 52% and a maximum moisture content of 65%. The pH was in the range 5.1 – 5.6.

The results obtained for the analysed samples are reported in tab. 5

TABLE 5 CHEMICAL COMPOSITION OF PDO MOZZARELLA CHEESE (MEAN VALUES ± STANDARD DEVIATIONS

Parameters	
pH	5.3 ± 0.1
Moisture (%)	56.6±0.2
Dry matter(%)	43.4±0.2
Protein(%)	17.5±0.1
Fat(%)	24.7±0.2
Fat/dry matter	56.8±0.8

7.2 Evaluation of the γ4-CN and αS1-CN effectiveness

Analysis of γ4-CN

The fragment f (69-209)-CN, deriving from the hydrolysis of β casein by Plasmin, also called γ4-CN, was reported by some authors (Di Luccia et al., 2009) as marker of frozen milk and curd in Buffalo Mozzarella PDO cheese. This fragment has a molecular weight of 15.748 kDa and it has no counterpart in bovine milk.

These authors reported the γ4-CN as a faint electrophoresis band in the fresh buffalo milk, which drastically increased in intensity in refrigerated and frozen milk as well as in frozen buffalo curds. Therefore it was suggested as a possible marker of freshness.

To assess the possibility to use this fragment as marker of frozen curd in mozzarella cheese, SDS-PAGE electrophoretic profiles were obtained for raw milk, thermized milk, premature curd, mature curd and mozzarella cheese in their fresh state and during a freezing storage of 9 months. Samples were analysed monthly. Samples collected in the three different dairies showed an identical behavior, so only results deriving from analysis of dairy A were reported.

The casein profiles of raw milk, fresh and frozen, are shown in fig. 9.

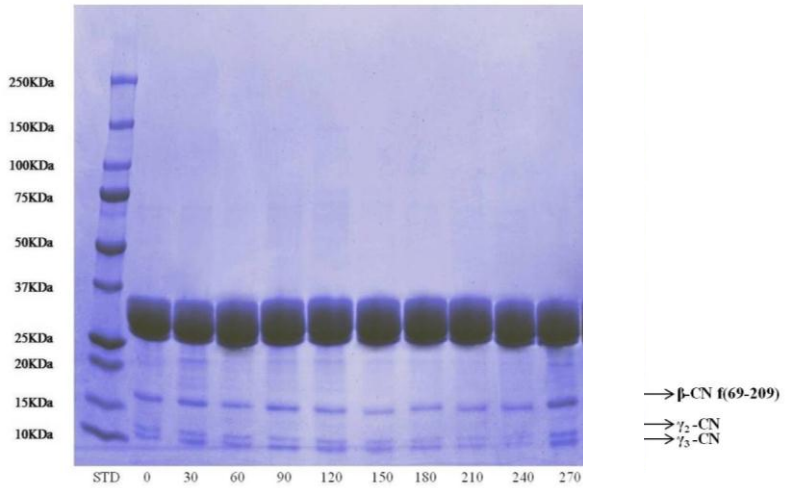


FIGURE 9 POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE) OF RAW MILK (RM) AT DIFFERENT FREEZING TIMES

The first lane of the gel shows the standard protein (STD) with molecular weights from 10 kDa to 250 kDa. In the second lane the electrophoretic profile of fresh raw milk is shown. Then, the profiles of milk after a freezing treatment for 30 to 270 days, are reported in the following lanes.

The profile of raw milk presented three electrophoretic bands: γ4-CN (f (69-209)), γ2-CN and γ3-CN, which derive from the hydrolysis of β-casein by the action of plasmin (Ismail & Nielsen, 2010).

The casein profiles of premature and mature curd (fresh and after freezing storage) are shown in fig. 10 and 11 respectively.

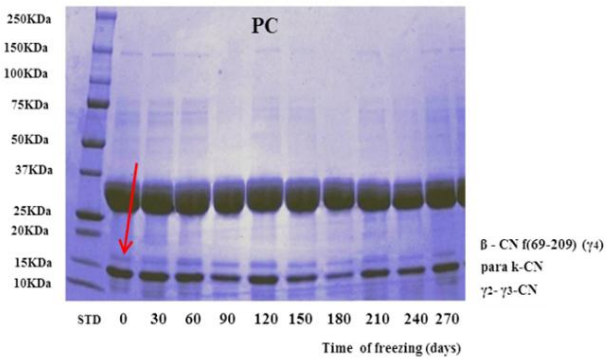


FIGURE 10 Polyacrylamide gel electrophoresis (SDS-PAGE) of premature curd (PC) at different freezing times

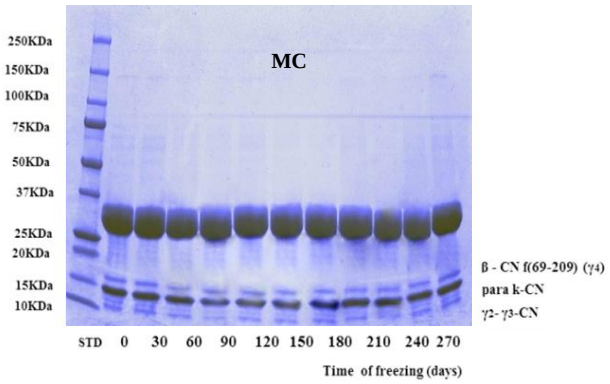


FIGURE 11 Polyacrylamide gel electrophoresis (SDS-PAGE) of mature curd (MC) at different freezing times

The same sequence was repeated for curd electrophoresis: lane 1 standard protein (STD) with molecular weights from 10 kDa to 250 kDa, lane 2 fresh curd and the following lanes show the casein profile of curd after freezing storage for 30 to 270 days.

The profiles of premature curd, mature curd and mozzarella showed an additional electrophoretic band respect to milk. Besides γ4-CN, γ2-CN and γ3-CN deriving from plasmin action, the para-k-CN was detected.

Our results show that the fragment γ4-CN is a band of identical intensity in all analysed samples (both fresh and frozen at different freezing times).

To quantify the amount of γ4-CN, a densitometric analysis was performed calculating the % of the normalized amount of γ4-CN compared to the β –CN for milk and to the para-k-CN for curd. The analysis confirmed that no significant differences ($p>0.05$) were found in the amount of γ4-CN between fresh and frozen samples (fig.12).

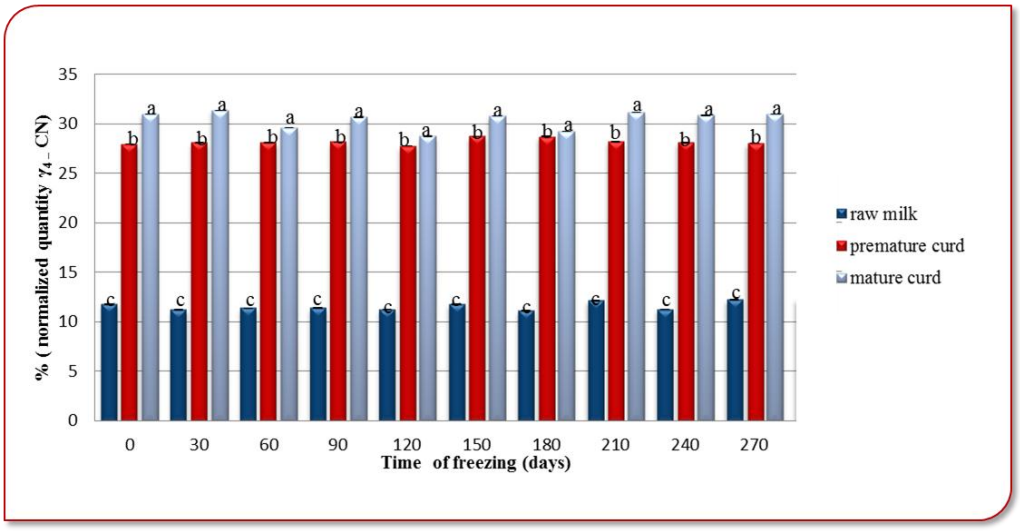


FIGURE 12 DETERMINATION OF Γ4-CN IN MILK AND CURDS.

a-c: different letters correspond to statistically significant differences (P<0.05)

In fig.13 the casein profiles of fresh samples and the correspondent samples at the end of experimental freezing period tested (9 months) are shown, to have a clear and complete scheme of all samples. In particular, the first lane of the gel shows the protein standard (STD) with molecular weights from 10 kDa to 250 kDa. In the second lane the profile of raw milk hydrolyzed by plasmin has been reported to clearly identify the production of the γ-CN fragment.

The following lanes show the electrophoretic profile of all fresh samples (t0) and the same samples frozen for 9 months (t9).

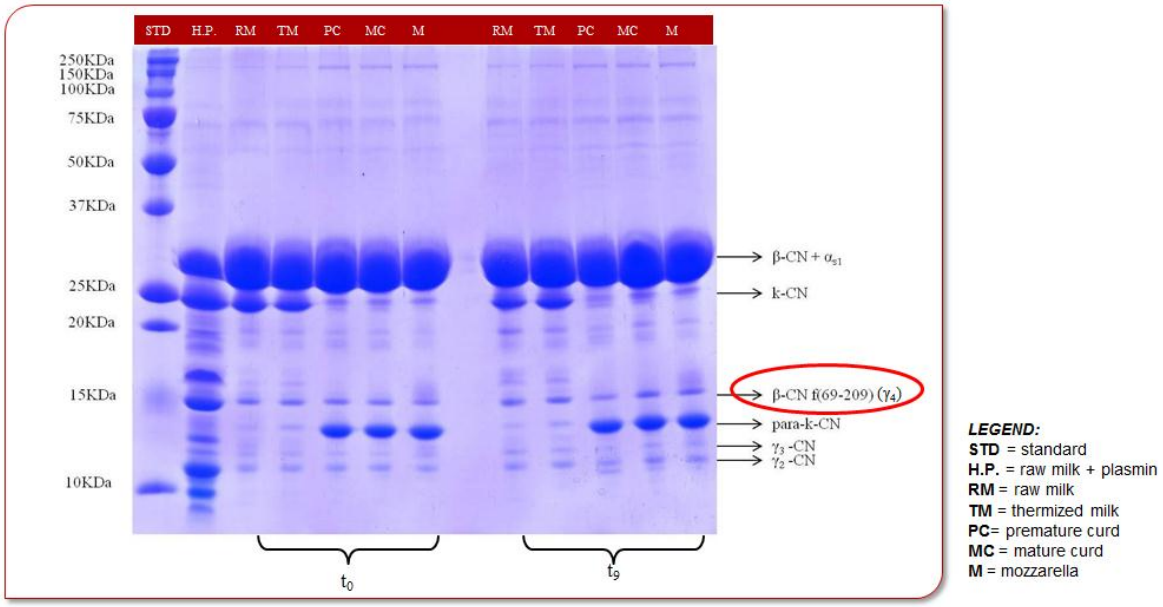


FIGURE 13 POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE) OF FRESH (T0) AND FROZEN (T9) SAMPLES

It can be noted that were plasmin was added into milk, a clear increase in γ4-CN fragment was obtained.

As previously described, Di Luccia *et al.* 2009 reported an increase in γ4-CN intensity in refrigerated and frozen buffalo milk. Our results showed, instead, a clear steady trend of γ4-CN amount during freezing storage of samples.

This discordant result can be explained by the proteolytic action of plasmin on β-casein, which appears more or less intense depending on many factors, such as the process conditions, animal health, phase and number of lactations (Burbrink & Hayes, 2006). The fresh milk used in this study showed quality and hygiene parameters within the limits provided by the current legislation.

To confirm if during the freezing storage the γ4-CN fragment might increase, a plasminolysis test was carried out. In particular, the production of the γ4-CN was tested in fresh and frozen raw milk (RM) with and without enzyme addition. Moreover, plasmin activity was tested in its ideal conditions to act (37°C x 1h). The obtained results are shown in fig.14. High concentrations of samples were loaded into the gel to visualize better the casein fragment of interest.

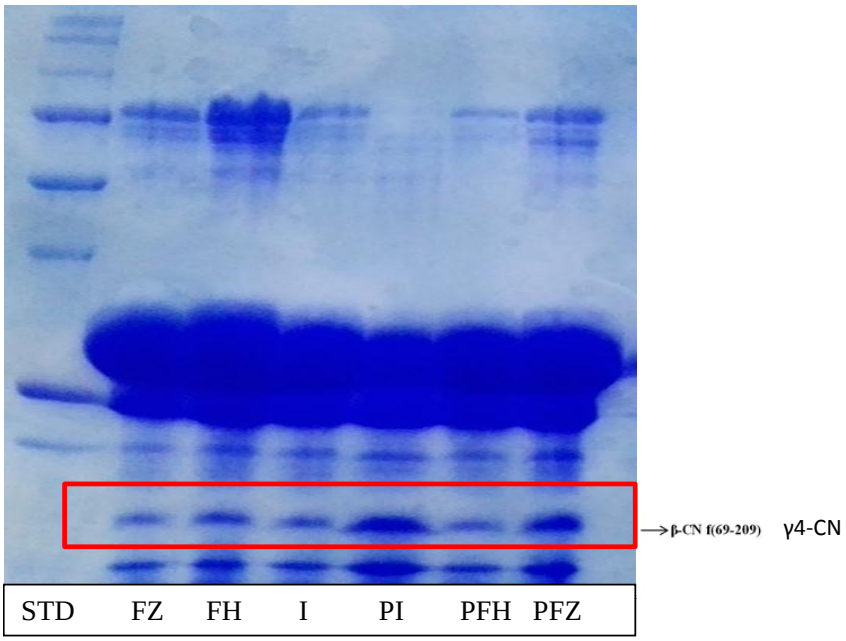


FIGURE 14PLASMINOLYSIS TEST IN RAW MILK. STD: STANDARD PROTEIN; FZ:FROZEN MILK; FH:FRESH MILK; I:FRESH MILK HELD IN IDEAL CONDITION OF PLASMIN ACTIONV(37°C X 1 H); PI; FRESH MILK ADDED WITH PLASMIN AND HELD IN IDEAL CONDITION OF PLASMIN ACTION (37°C X 1 H); PFH:FRESH MILK ADDED WITH PLASMIN; PFZ: FROZEN MILK ADDED WITH PLASMIN

As it can be observed, where plasmin was added, an increase in γ4-CN intensity was obtained when the sample was held in the ideal condition for plasmin activity (sample PI) . In fact a more intense band correspondent to γ4-CN was detected in the sample PI respect to the sample I.

In the samples where no plasmin was added, freezing had no effect on fragment increase, while where the enzyme was added, a more intense fragment was observed after freezing (PFZ) respect to the fresh sample (PFH). Maybe the more intense plasmin action, where plasmin was added, was due to the presence of a higher concentration of the enzyme.

In fact, enzyme activity is reported as affected by concentration of enzyme, concentration of substrate, pH and temperature. Being equal the concentration of substrate, pH and temperature if the concentration of the substrate is constant and the concentration of the enzyme increases, the rate of reaction is reported to increase linearly (Bettelhaim et al., 2015).

In our experimental conditions, the plasmin amount was the only difference between the group of samples PI-PFH-PFZ (with plasmin addition) and the group of samples I-FH-FZ (without plasmin addition).

Analysis of αS1-CN

The αS1-CN fragments, deriving from the action of the exogenous enzyme chymosin, were suggested by some authors (Petrella et al., 2015) as possible markers of freshness in buffalo mozzarella cheese. They detected an abnormal hydrolysis of αS1-CN resulting in αS1 casein fragments in stored buffalo milk and curd.

To check the effectiveness of αS1-CN fragments in the evaluation of freshness of buffalo mozzarella PDO cheese, the UREA PAGE was used for the analysis of casein profiles. Fresh raw milk, thermized milk, premature curd, mature curd, mozzarella cheese and the corresponding frozen samples were analysed monthly, for a total period of 9 months of freezing storage..

The casein profiles of fresh samples and samples stored for 9 months at -20°C are shown in fig. 15.

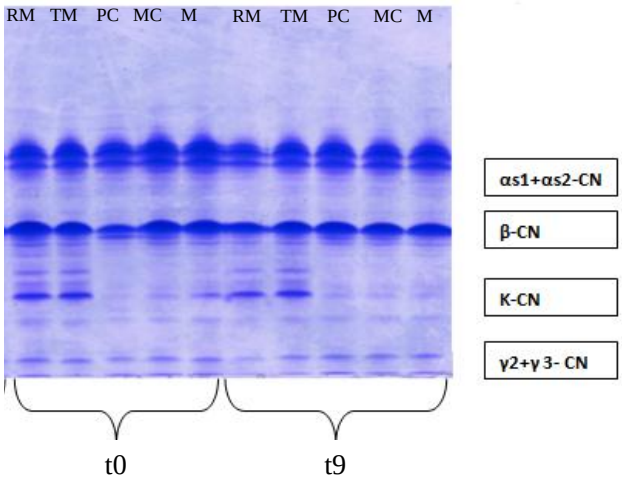


FIGURE 15 UREA PAGE OF FRESH (T0) AND FROZEN (T9) SAMPLES. RM:RAW MILK; TM:THERMIZED MILK; PC:PREMATURE CURD; MC:MATURE CURD; M: MOZZARELLA

Milk hydrolyzed by external addition of chymosin was used as positive control (lane 1 in fig. 16)

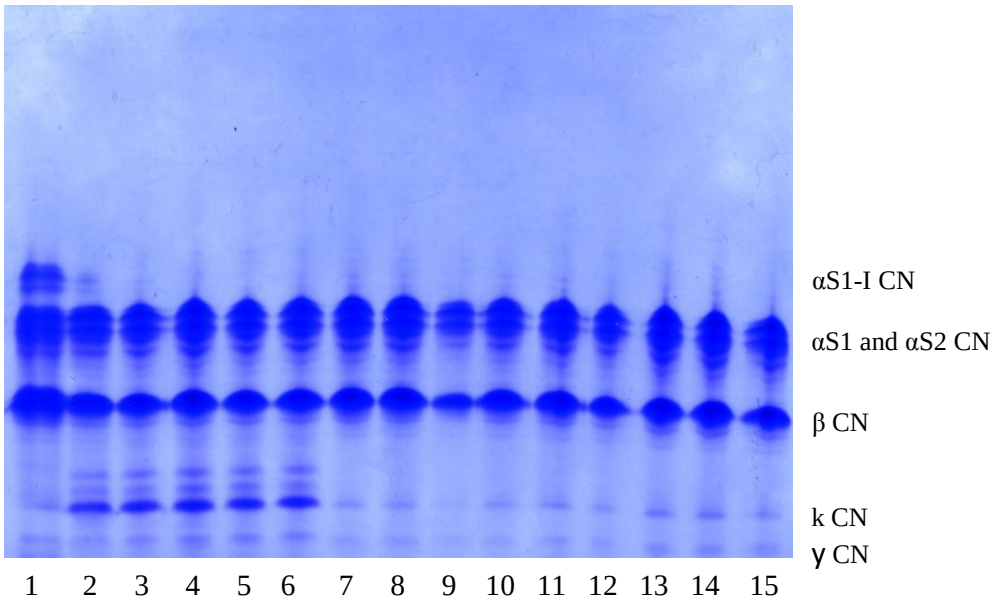


FIGURE 16 UREA-PAGE PATTERN OF FROZEN MILK, CURD AND MOZZARELLA CHEESE. LANE 1: MILK HYDROLYZED WITH CHYMOSIN; LANES 2-4: FROZEN RAW MILK; LANES 5-6: FROZEN THERMIZED MILK; LANES 7-9; FROZEN PREMATURE CURD; LANES 10-12: FROZEN MATURE CURD; LANES 13-15: FROZEN MOZZARELLA CHEESE

The casein profiles obtained by Urea Page of milk, semi-finished products and mozzarella cheese showed an absolute identity between the fresh and frozen state of the samples. The αs1-I casein fragments were not detected in analysed samples.

The presence of αs1-I casein fragments was also evaluated in buffalo mozzarella cheese obtained from 100% frozen curd, produced with curd stored for 6 months at -20°C. The analysis of fresh buffalo mozzarella cheese and mozzarella cheese obtained from 100% frozen curd confirmed the absence of those fragments. Stored mozzarella cheese produced from cow milk (fiordilatte) was examined as positive control for the presence of the fragments, as indicated by Faccia et al. (2014).

These authors reported that the as1-I casein fragment (f 24-199) was the main product of primary proteolysis formed by the action of chymosin on as1 casein, during the first part of cheesemaking process. According to these authors the fragment can be easily detected by electrophoresis in the presence of urea (urea-PAGE).

In the fig. 17, the same casein profiles can be observed for fresh and frozen curd and for buffalo mozzarella produced by the use of those curds. A different profile can be observed for fiordilatte, where the αs1-I CN resulted visible (lane 5).

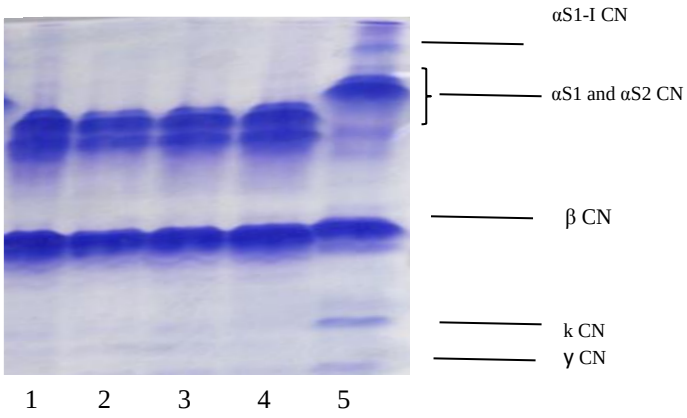


FIGURE 17 UREA PAGE 1: BUFFALO MOZZARELLA CHEESE OBTAINED FROM 100% FROZEN CURD; 2:FROZEN CURD; 3:FRESH BUFFALO MOZZARELLA CHEESE; 4: FRESH CURD; 5: COW MOZZARELLA CHEESE

According to our results, αs1-I CN cannot be used to verify conformity to PDO, since the same casein profile was observed in fresh and frozen samples and in fresh mozzarella cheese and mozzarella cheese produced from frozen curd.

As reported by Faccia et al (2014), under normal conditions the formation of that fragment is not detectable. It could be possible that, in our case, a high quality of raw material and a controlled process, avoided conditions for the development of an intense proteolysis.

7.3 Furosine content

Determination of furosine is used to evaluate the extent of the early Maillard reaction in milk products. It is formed by hydrolysis of the main stable Amadori

compound and it is used as indicator of heat damage of milk, enabling distinction of UHT milk, pasteurized milk and sterilized milk.
The Maillard reaction caused by heat treatment leads to the decrease in nutritional value of proteins and formation of brown compounds in milk (Claeys et al., 2001).
Resmini et al. (1992) reported an increase in furosine also when milk is stored at freezing temperature (-20°C). they found an increase of furosine of 1 mg every 30 days of storage.
With the purpose to verify if furosine could be a marker of freezing of dairy products, it was evaluated in fresh and stored milk, curd and cheese.

The furosine content of fresh and frozen curd at different freezing time has been reported in fig. 18.

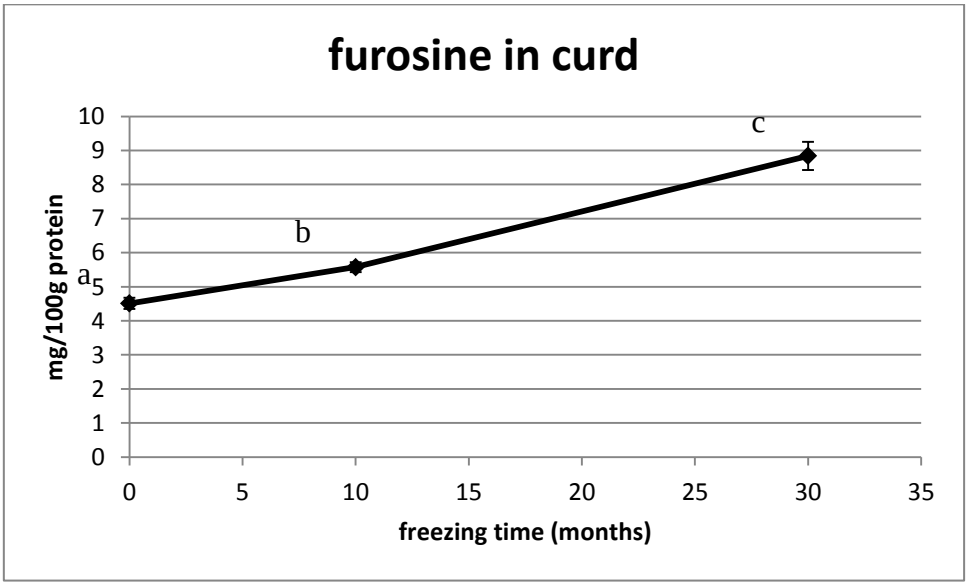


FIGURE 18 FUROSINE CONTENT IN FRESH (TIME 0) AND FROZEN CURD

a-c: different letters correspond to significant differences ($p<0.05$)

Notwithstanding a significant increase was detected during 30 months of storage at -20°C, the value rose less than 1mg/100g protein. In two years and half, an increase of approximately 4.5 mg/100g protein was detected.

In fresh mozzarella cheese, a content of 5.90 ±0.24 mg/100g was found. After 30 months of storage, a significant increase was detected 9.90 ±0.63.

7.4 Lipids analysis

7.4.1 Triglycerides profile

The triglycerides (TG) profiles of fresh and stored milk and curd are reported in tab. 6 and 7.

TG families from C24 to C 54 were detected in every sample. The main triglycerides group were represented by C36 and C38, with percentage of about 14% on the total fat content.

The TG qualitative and quantitative profiles of milk, curd and mozzarella cheese resulted very similar.

TABLE 6 TRIGLYCERIDES COMPOSITION OF BUFFALO MILK (MEAN VALUE ± SD)

Milk			
	TIME (days)		
% on total fat	0	120	270
C24	1.54±0.16	1.74±0.24	1.82±0.42
C26	2.15±0.47	2.28±0.27	2.41±0.17
C28	2.11±0.35	2.34±0.45	2.45±0.37
C30	2.41±0.35	2.43±0.42	2.59±0.47
C32	3.61±0.41	3.28±0.37	3.59±0.24
C34	8.15±0.16	8.37±0.35	8.24±0.35
C36	14.11±0.12	14.2±0.32	14.09±0.35
C38	14.62±0.35	14.61±0.22	14.69±0.41
C40	9.39±0.48	9.6±0.48	9.07±0.16
C42	5.47±0.42	5.38±0.35	5.46±0.36
C44	5.22±0.37	5.29±0.22	5.16±0.47
C46	6.99±0.31	6.91±0.34	6.83±0.42
C48	8.96±0.46	8.73±0.34	8.73±0.11
C50	8.39±0.11	8.23±0.41	8.16±0.35
C52	5.42±0.35	5.27±0.16	5.27±0.25
C54	1.51±0.44	1.34±0.22	1.45±0.22

(p>0.05)

TABLE 7 TRIGLYCERIDES COMPOSITION OF CURD (MEAN VALUE ± SD)

Curd			
	TIME (days)		
% on total fat	0	120	270
C24	2.29±0.25	1.90±0.42	2.00±0.42
C26	2.59±0.36	2.53±0.17	2.59±0.17
C28	2.86±0.27	2.40±0.36	2.71±0.37
C30	2.71±0.45	2.60±0.36	2.66±0.22
C32	3.10±0.42	3.72±0.36	3.58±0.34
C34	8.11±0.37	8.33±0.48	8.07±0.16
C36	13.12±0.31	13.79±0.35	13.41±0.35
C38	13.03±0.22	13.84±0.23	13.55±0.41
C40	9.43±0.34	9.04±0.12	8.8±0.16
C42	5.26±0.16	5.09±0.35	5.18±0.36
C44	5.36±0.47	5.11±0.48	4.92±0.47
C46	6.76±0.46	6.41±0.16	7.16±0.42
C48	10.13±0.11	8.92±0.23	10.04±0.11
C50	9.02±0.35	9.25±0.12	9.2±0.35
C52	4.97±0.35	5.71±0.26	4.82±0.25
C54	1.26±0.44	1.38±0.22	1.31±0.22

(p>0.05)

As can be observed, and confirmed by statistical analysis, no significant differences were detected between fresh and frozen samples during 9 months of freezing storage.

7.4.2 Fatty acid profile

Fatty acids from C4 to C22 were detected in all samples. In fig. 19, the fresh and frozen (9 months) fatty acid profiles of milk, curd and mozzarella cheese overlaid. Concentration of saturated fatty acids (approximately 70%), monounsaturated fatty acids (approximately 26%) and polyunsaturated fatty acids (3%) were found. Only the main fatty acids are reported in the figure.

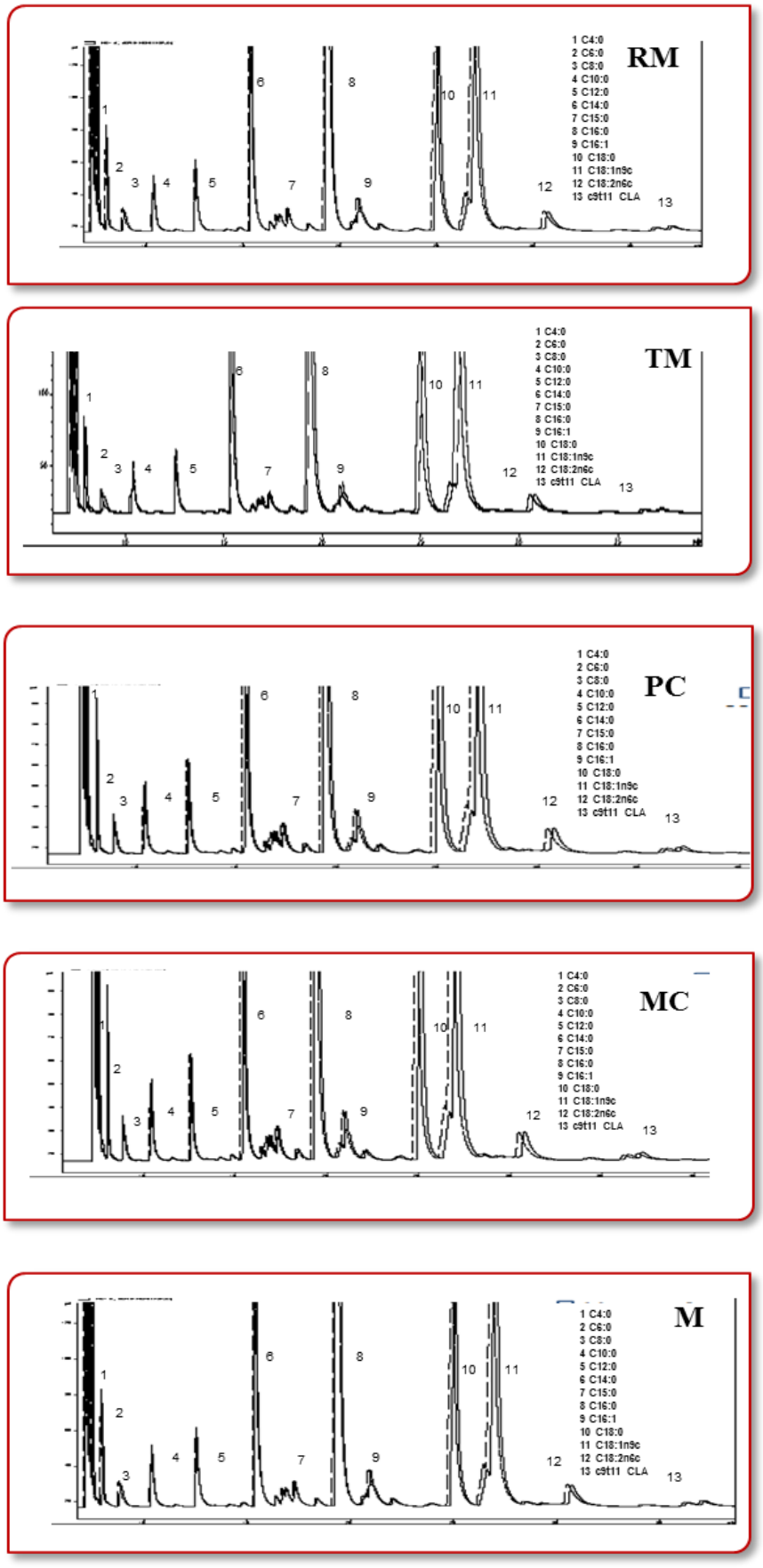


FIGURE 19 FATTY ACIDS PROFILES OF RAW MILK (RM), THERMIZED MILK (TM), PREMATURE CURD (PC), MATURE CURD (MC) AND MOZZARELLA CHEESE (M) IN THEIR FRESH STATE (CONTINUOUS LINE) AND AFTER 270 DAYS OF FREEZING (DOT LINE)

As observed and confirmed by the statistical analysis, no significant differences (p>0.05) between the fresh and frozen fatty acid profiles were detected. The complete lists of fatty acids identified in fresh and frozen samples are reported in tab. 8-11.

TABLE 8 FATTY ACID COMPOSITION (G/100 G) OF FRESH (T0) AND FROZEN MILK (T9) (MEAN VALUES ± STANDARD DEVIATIONS)

FA	t0	t9
C4:0	1.81 ±0.16	2.39 ±0.05
C6:0	1.79 ±0.21	2.01 ±0.09
C8:0	0.93 ±0.02	1.05 ±0.00
C10:0	1.97 ±0.01	1.66 ±0.16
C11:0	0.09 ±0.00	0.07 ±0.00
C12:0	2.49 ±0.00	2.48 ±0.01
C13:0	0.10 ±0.00	0.09 ±0.00
C14:0	10.55 ±0.06	10.40 ±0.06
C14:1	0.84 ±0.00	0.81 ±0.01
C15:0	1.07 ±0.00	1.06 ±0.01
C16:0	34.97 ±0.17	34.43 ±0.15
C16:1	1.28 ±0.03	1.18 ±0.02
C17:0	0.98 ±0.02	1.06 ±0.03
C17:1	0.44 ±0.00	0.47 ±0.00
C18:0	13.25 ±0.04	13.20 ±0.06
C18:1n9t	1.47 ±0.00	1.36 ±0.02
C18:1n9c	21.88 ±0.06	22.03 ±0.15
C18:2n6t	0.13 ±0.00	0.11 ±0.01
C18:2n6c	2.35 ±0.00	2.53 ±0.16
C20:0	0.28 ±0.00	0.28 ±0.02
C20:1	0.56 ±0.01	0.62 ±0.07
c9t11CLA	0.21 ±0.00	0.22 ±0.00
C18:3n3	0.03 ±0.00	0.03 ±0.00
C20:2	0.02 ±0.00	0.01 ±0.00
C20:3n3	0.08 ±0.00	0.09 ±0.01
C20:4n6	0.09 ±0.02	0.05 ±0.00
C20:5n3	0.06 ±0.00	0.07 ±0.00
C22:6n3	0.05 ±0.00	0.04 ±0.01
C22:0	0.04 ±0.00	0.03 ±0.00
C22:2	0.14 ±0.00	0.15 ±0.00

(p>0.05)

TABLE 9 FATTY ACID COMPOSITION (G/100 G) OF FRESH (T0) AND FROZEN PREMATURE CURD (T9) (MEAN VALUES ± STANDARD DEVIATIONS)

FA	t0	t9
C4:0	2.12 ±0.12	2.15 ±0.01
C6:0	2.07 ±0.08	2.04 ±0.07
C8:0	1.03 ±0.01	1.01 ±0.02
C10:0	2.07 ±0.00	2.09 ±0.03
C11:0	0.10 ±0.00	0.09 ±0.00
C12:0	2.53 ±0.01	2.53 ±0.02
C13:0	0.10 ±0.00	0.10 ±0.00
C14:0	10.60 ±0.01	10.55 ±0.08
C14:1	0.83 ±0.02	0.83 ±0.01
C15:0	1.05 ±0.02	1.06 ±0.01
C16:0	34.75 ±0.07	34.61 ±0.01
C16:1	1.24 ±0.03	1.24 ±0.04
C17:0	0.94 ±0.01	1.04 ±0.04
C17:1	0.44 ±0.00	0.47 ±0.01
C18:0	13.08 ±0.07	12.96 ±0,10
C18:1n9t	1.41 ±0.03	1.43 ±0.05
C18:1n9c	21.64 ±0.00	21.84 ±0.01
C18:2n6t	0.13 ±0.00	0.11 ±0.00
C18:2n6c	2.32 ±0.01	2.34 ±0.02
C20:0	0.28 ±0.00	0.27 ±0.01
C20:1	0.57 ±0.02	0.57 ±0.01
c9t11CLA	0.20 ±0.01	0.21 ±0.01
C18:3n3	0.02 ±0.00	0.03 ±0.00
C20:2	0.02 ±0.00	0.02 ±0.00
C20:3n3	0.15 ±0.00	0.14 ±0.00
C20:4n6	0.09 ±0.00	0.09 ±0.00
C20:5n3	0.07 ±0.02	0.04 ±0.00
C22:6n3	0.07 ±0.01	0.07 ±0.02
C22:0	0.05 ±0.00	0.04 ±0.00
C22:2	0.03 ±0.01	0.04 ±0.00

(p>0.05)

TABLE 10 FATTY ACID COMPOSITION (G/100 G) OF FRESH (T0) AND FROZEN MATURE CURD (T9) (MEAN VALUES ± STANDARD DEVIATIONS)

FA	t0	t9
C4:0	2.04 ±0.09	2.43 ±0.16
C6:0	1.98 ±0.06	2.21 ±0.07
C8:0	1.02 ±0.02	1.15 ±0.10
C10:0	2.03 ±0.01	2.26 ±0.18
C11:0	0.09 ±0.01	0.09 ±0.01
C12:0	2.52 ±0.01	2.69 ±0.18
C13:0	0.10 ±0.00	0.10 ±0.01
C14:0	10.47 ±0.1	10.97 ±0.54
C14:1	0.84 ±0.00	0.88 ±0.06
C15:0	1.06 ±0.01	1.09 ±0.04
C16:0	34.49 ±0.35	34.75 ±0.21
C16:1	1.23 ±0.01	1.24 ±0.02
C17:0	0.97 ±0.01	1.09 ±0.01
C17:1	0.44 ±0.01	0.46 ±0.01
C18:0	13.11 ±0.13	12.17 ±0.75
C18:1n9t	1.45 ±0.00	1.34 ±0.01
C18:1n9c	22.04 ±0.38	21.35 ±0.58
C18:2n6t	0.14 ±0.00	0.11 ±0.01
C18:2n6c	2.43 ±0.06	2.28 ±0.06
C20:0	0.29 ±0.01	0.24 ±0.04
C20:1	0.58 ±0.02	0.54 ±0.02
c9t11CLA	0.21 ±0.01	0.20 ±0.01
C18:3n3	0.02 ±0.00	0.02 ±0.01
C20:2	0.02 ±0.00	0.02 ±0.01
C20:3n3	0.15 ±0.00	0.12 ±0.02
C20:4n6	0.09 ±0.00	0.04 ±0.00
C20:5n3	0.08 ±0.01	0.04 ±0.01
C22:6n3	0.06 ±0.00	0.05 ±0.01
C22:0	0.05 ±0.00	0.03 ±0.01
C22:2	0.03 ±0.00	0.02 ±0.01

(p>0.05)

TABLE 11 FATTY ACID COMPOSITION (G/100 G) OF FRESH (T0) AND FROZEN MOZZARELLA CHEESE (T9) (MEAN VALUES ± STANDARD DEVIATIONS)

FA	t0	t9
C4:0	2.30 ±0.36	2.06 ±0.06
C6:0	2.04 ±0.42	2.21 ±0.09
C8:0	1.03 ±0.15	1.08±0,04
C10:0	2.15 ±0.15	2.11 ±0.04
C11:0	0.09 ±0.01	0.09 ±0.01
C12:0	2.61 ±0.14	2.53 ±0.02
C13:0	0.11 ±0.00	0.10 ±0.00
C14:0	10.83 ±0.32	10.53 ±0.01
C14:1	0.86 ±0.02	0.85 ±0.02
C15:0	1.08 ±0.02	1.05 ±0.01
C16:0	34.96 ±0.04	34.51 ±0.31
C16:1	1.25 ±0.04	1.28 ±0.02
C17:0	0.98 ±0.05	1.03 ±0.01
C17:1	0.44 ±0.01	0.47 ±0.01
C18:0	12.66 ±0.70	12.59 ±0.05
C18:1n9t	1.38 ±0.01	1.26 ±0.12
C18:1n9c	21.31 ±0.70	22.32 ±0.39
C18:2n6t	0.13 ±0.00	0.11 ±0.00
C18:2n6c	2.31 ±0.05	2.33 ±0.05
C20:0	0.26 ±0.04	0.27 ±0.01
C20:1	0.56 ±0.03	0.58 ±0.01
c9t11CLA	0.21 ±0.01	0.22 ±0.01
C18:3n3	0.02 ±0.01	0.02 ±0.00
C20:2	0.02 ±0.00	0.01 ±0.00
C20:3n3	0.08 ±0.00	0.04 ±0.00
C20:4n6	0.07 ±0.00	0.04 ±0.01
C20:5n3	0.05 ±0.01	0.07 ±0.01
C22:6n3	0.04 ±0.01	0.04 ±0.00
C22:0	0.02 ±0.00	0.03 ±0.00
C22:2	0.13 ±0.02	0.15 ±0.00

(p>0.05)

Our results of fatty acid profiles are consistent with those of Zhang et al (2006), where no effect on the milk or cheese fatty acid composition was found after the freezing storage.

7.4.3 Lipolysis and oxidation

The analysis of FFA showed no significant differences between fresh and frozen curd, while an increase in FA in mozzarella cheese was detected. The results are reported in tab.12.

TABLE 12 FFA (% OLEIC ACID) IN MATURE CURD (MC) AND MOZZARELLA CHEESE (M) AFTER 9 MONTHS OF FREEZING STORAGE

	t0	t9
MC	0.75 [a] ±0.02	0.73 [a] ±0.01
M	0.62 [a] ±0.03	0.74 [b] ±0.02

a-b: different letters in the same raw correspond to statistical significant differences

PV showed no statistical differences between fresh and frozen samples. The values detected in mature curd and mozzarella cheese stored for 9 months are reported in tab. 13. A decrease of nearly 7 % was observed in frozen curd, while no significant difference was observed in mozzarella cheese.

TABLE 13 PV VALUE (meqO2/kg) IN MATURE CURD (MC) AND MOZZARELLA CHEESE (M) AFTER 9 MONTHS OF FREEZING STORAGE

	t0	t9
MC	0.72 [a] ±0.03	0.67 [b] ±0.01
M	0.63 [a] ±0.02	0.65 [a] ±0.03

a-b: different letters in the same raw correspond to statistical significant differences

Mono-diglycerides (MDG) were examined as an index of lipolytic activity because the lipases can attack the globules of milk fat, release free fatty acids and form mono- and diglycerides from triglycerides (TG) (Metha, 2015). According to Bareth et al (2003) if the sample contains fat, the diglycerides detection can be interfered by the presence of triglycerides and fatty acids from C4 to C14 and diglycerides cannot be chromatographically separated. To avoid these possible interferences, TG and phospholipids (P) were separated by solid phase extraction (SPE). No significant difference between fresh and frozen mature curd was detected. The MDG qualitative profiles for fresh and frozen curd after a period of 9 months were identical. After a freezing period of 9 months, an increase of 5.2% and 8.5% was revealed for 1,2-Dipalmitin and 1,3-Diolein, respectively. However, the obtained results cannot be considered markers of freezing storage.

The quantification results of MDG, TG and P in mature curd showed no significant differences between fresh and frozen samples. The results are shown in tab. 14.

TABLE 14 COMPOSITION (%) OF TG, MDG AND P OF FAT EXTRACTED BY FRESH (T0) AND FROZEN (T9) MATURE CURD (MC). MEAN VALUES ± STANDARD DEVIATION

	TG	MDG	P
MC- t0	95.9 ±0.6	4.0 ±0.6	0.2 ±0.0
MC- t9	95.8 ±0.5	3.9 ±0.6	0.3 ±0.1

TG: triglycerides, MDG: Mono-diglycerides, P:Phospholipids

No statistical significant difference in the same column (p<0.05)

In fig. 20, the MDG profiles of fresh and frozen mature curd are reported.

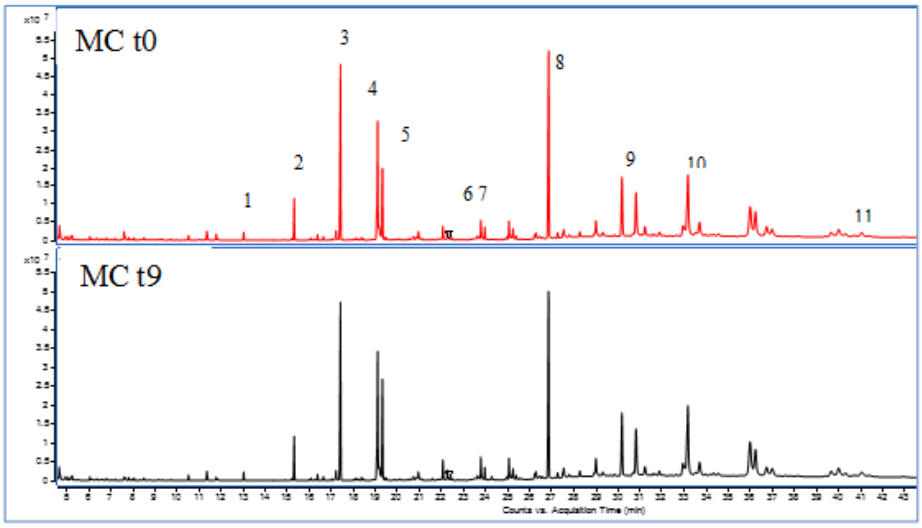


FIGURE 20 FRESH (T0) AND FROZEN (9 MONTHS) MATURE CURD MDG PROFILE

Note - 1:Decanoic acid, TMS derivative; 2:Myristic acid, TMS derivative; 3: Palmitic acid, TMS derivative; 4:Oleic acid, TMS derivative; 5:Stearic acid, TMS derivative; 6:1-Monoolein, 2TMS derivative; 7: 1-Monopalmitin, 2TMS derivative; 8: Cholesterol, TMS derivative; 9: Internal standard (betulin); 10:1,2-Dipalmitin, TMS derivative; 11: 1,3-Dipalmitin, TMS derivative

Among the identified molecules, significant differences were detected for 1,2-Dipalmitin and 1,3-Diolein between fresh and the frozen curd. In tab. 15, the detected amounts are reported.

TABLE 15 1,2-DIPALMITIN AND 1,3-DIOLEIN CONTENT IN FRESH AND FROZEN CURD (9 MONTHS). THE RESULTS ARE REPORTED AS % OF TOTAL MDG (AVERAGE VALUES ± STANDARD DEVIATIONS)

	fresh	frozen
1,2-Dipalmitin	3.61^{a}±0.04	3.80^{b}±0.06
1,3-Diolein	1.17^{a}±0.01	1.27^{b}±0.01

a-b: Different letters in the same row correspond to statistical significant differences ($p<0.05$)

7.4.4 Thermal profile analysis

Differential scanning calorimetry is a technique which can be used to study food quality and authenticity. It was used for distinction of mozzarella cheese from imitation mozzarella cheese made from calcium caseinate (Tunick and Malin, 1997). In that case, the enthalpy of the milk fat melting transition decreased with increase in caseinate concentration. Those results were confirmed by scanning electron microscope (SEM) where an agglomeration of lipids in imitation samples was observed, whereas natural cheese showed a uniform dispersion of fat globules. Addition of caseinate affected fat crystallization, leading to an enthalpy reduction (Singhal & Kulkarni 1997). DSC was also used by some authors to discriminate mozzarella cheese made by cow and buffalo milk (Tunick 2015).

The thermal behavior of anhydrous milk fat extracted from curds was studied. The fig. 21 and 22 compare the typical curves of fat extracted from fresh and frozen premature curd and mature curd respectively.

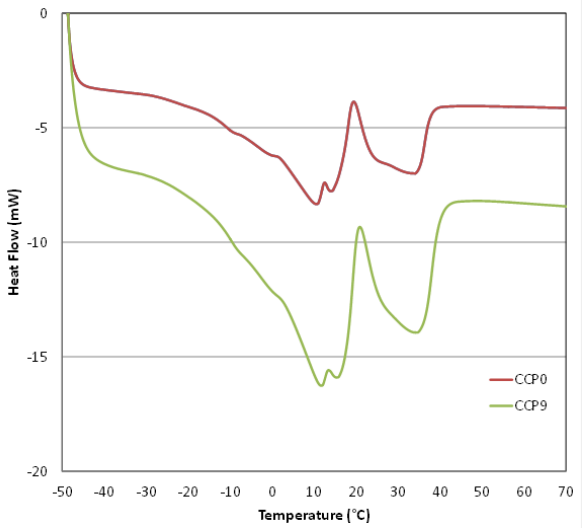


FIGURE 21 DIFFERENTIAL SCANNING CALORIMETRIC MELTING THERMOGRAMS OF FRESH (CCP0) AND FROZEN PREMATURE CURD (CCP9)

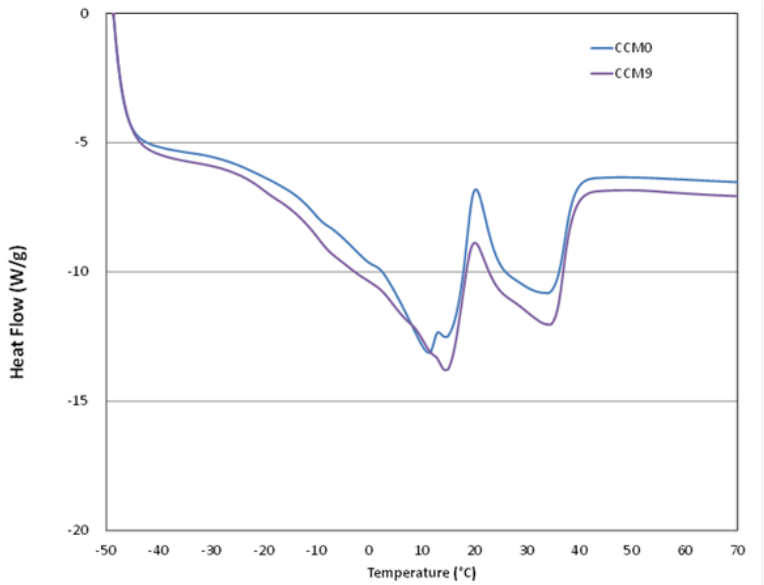


FIGURE 22 DIFFERENTIAL SCANNING CALORIMETRIC MELTING THERMOGRAMS OF FRESH (CCM0) AND FROZEN MATURE CURD (CCM9)

The fat in curd, as reported for mozzarella cheese by Tunick (2015), was found to melt from -40 to +40 °C. Both premature and mature curd, fresh and frozen, showed in fact a low- and high-temperature melting regions, which were separated by a valley in the curves at about 20°C: the first region (peak 1) between 10 and 15°C and the second region (peak 2) between 30 and 40°C.

The melting below 30°C is associated to triglycerides with low molecular weight (TG containing butyric and caproic acid esterified at position 3 on the glycerol molecule) and unsaturated triglycerides with high molecular weight (TG containing oleic acid at position 3) (Tunick and Malin, 1997, Tunick 2015). The high melting region is, instead, associated to triglycerides with saturated fatty acids.

The enthalpy values (ΔH) and peak temperatures of the fat extracted from mature curd (MC) and premature curd (PC) are listed in tab. 16. Values obtained for fresh (t0) and frozen (t9) samples are reported.

TABLE 16 THERMAL ANALYSIS OF FRESH AND STORED CURD (MEAN VALUES ± STANDARD DEVIATIONS)

	Peak 1		Peak 2		
	Tp (°C)	**ΔH (J/g)**	**Tp (°C)**	**ΔH (J/g)**	**ΔH (J/g) Tot.**
MC t0	11.38 ± 0.15	47.16^{b} ± 2.13	33.99 ± 0.08	20.04^{b} ± 0.84	**66.24^{b} ± 4.75**
MC t9	13.97 ± 0.45	30.71^{a} ± 0.33	34.3 ± 0.32	15.80^{a} ± 0.33	**46.51^{a} ± 0.00**
PC t0	10.61 ± 0.05	54.60^{c} ± 0.84	33.85 ± 0.13	24.02^{c} ± 0.43	**78.61^{c} ± 1.27**
PC t9	11.17 ± 0.01	44.15^{a} ± 0.76	34.57 ± 0.18	20.43^{b} ± 0.64	**64.58^{b} ± 1.40**

a-c: Different letters in the same column correspond to statistical significant differences ($p<0.05$)

Significant differences were observed in MC and PC enthalpy after freezing storage. In particular, a reduction of ΔH was detected in both cases and for both peaks.

The total enthalpy showed a decrease of about 30% and 18% in MC and PC respectively after 6 months of storage at -20°C.

It is known that fat crystallization can be influenced by the presence of minor lipids (acylglycerols, free fatty acids and phospholipids). In fact, polar lipids have been used historically to manipulate fat crystallization. Diacylglycerols can cocrystallize with triacylglycerols and stabilize different forms of crystals with different thermodynamic properties, even if the effects on physical properties depend on the chemical structure of minor lipids (Wright et al., 2000).

During freezing an increase in diolein and dipalmitin was observed in curd after 6 months of storage that could be associated to an influence of fat thermal properties.

Phase II: Evaluation of the effects of frozen curd addition in Buffalo Mozzarella cheese

7.5 Basic parameters, fat and protein composition of mozzarella cheese

Mozzarella cheese produced from additional percentage of frozen curd, from 0 (fresh) to 100% showed an average pH of 5.36 ± 0.23. These values were within a normal pH range for mozzarella cheese (5.1-5.6).

The chemical composition of mozzarella cheeses was reported in tab. 17.

TABLE 17 COMPOSITION OF MOZZARELLA FROM FRESH AND INCREASING PERCENTAGE OF FROZEN CURD

	frozen curd addition (%)				
Parameters (%)	**0**	**20**	**40**	**80**	**100**
Moisture	56.6±0.2	55.2±0.0	54.0±0.1	53.0±0.3	52.2±0.5
Fat	24.7±0.2	23.5±0.1	21.8±0.2	16.0±0.1	10.7±0.7
Protein	17.5±0.1	17.7±0.1	22.7±0.1	29.9±0.1	36.4±0.1
Dry Matter (DM)	43.4±0.2	44.8±0.1	46.0±0.2	47.0±0.3	47.8±0.5
Fat/DM	56.8±0.8	52.3±0.2	47.3±0.7	34.0±0.8	22.4±1.2

When the percentage of frozen curd increased, a reduction of moisture and fat content in Mozzarella cheese was observed, while the protein content increased (fig. 23 and 24).

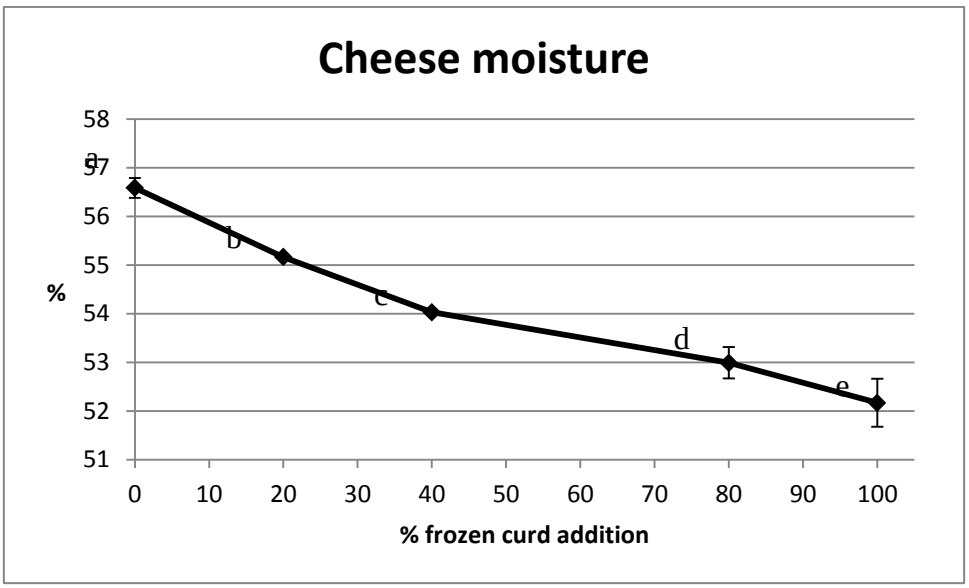


FIGURE 23 MOZZARELLA CHEESE MOISTURE AT INCREASING ADDITION OF FROZEN CURD

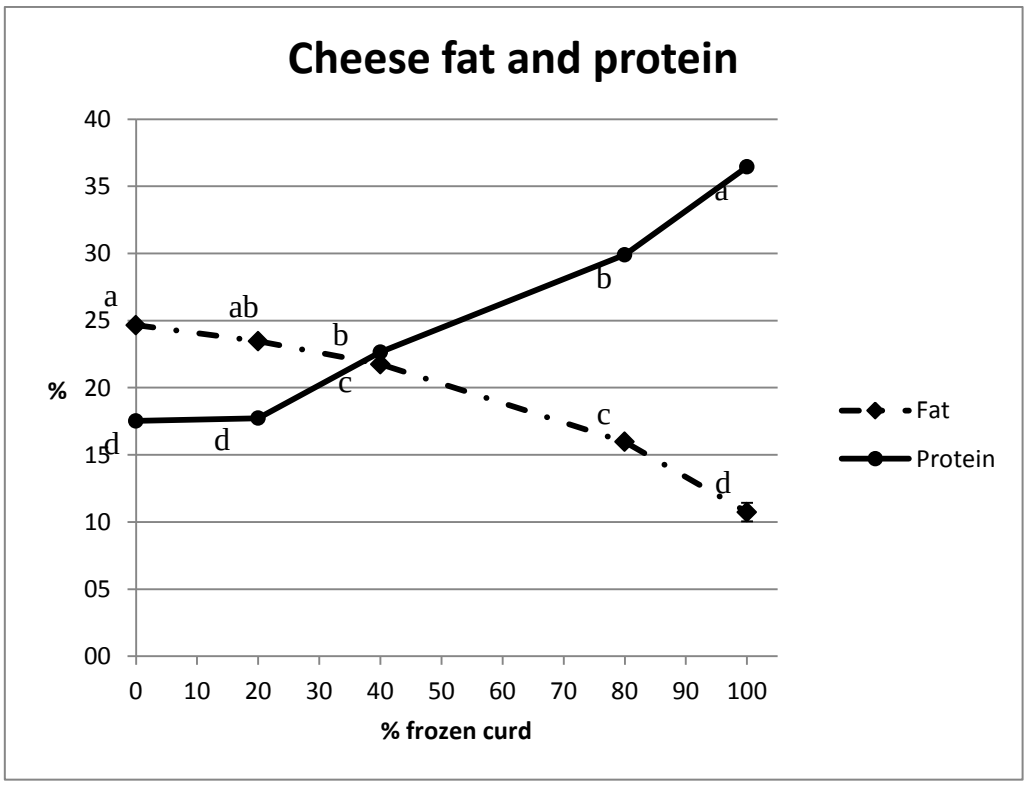


FIGURE 24 FAT YIELD IN MOZZARELLA CHEESE AT DIFFERENT ADDITION OF FROZEN CURD

a-d: different letters for the same parameter correspond to significant differences ($p<0.05$)

Fresh mozzarella cheese and mozzarella cheese produced with 20% frozen curd showed no significant differences in protein content, while a significant reduction of fat content, nearly 5%, was observed.

According to PDO specification, fat/dry matter ratio has to be >52%. The fat reduction and the increase in dry matter, due to loss of moisture, lead to decrease of this ratio with addition of fresh curd. However, mozzarella produced from 20% frozen curd showed parameters above the limit as fresh mozzarella cheese. In mozzarella cheese produced from percentage addition of 40% or more, the ratio decreased under the limit specified. (fig. 25)

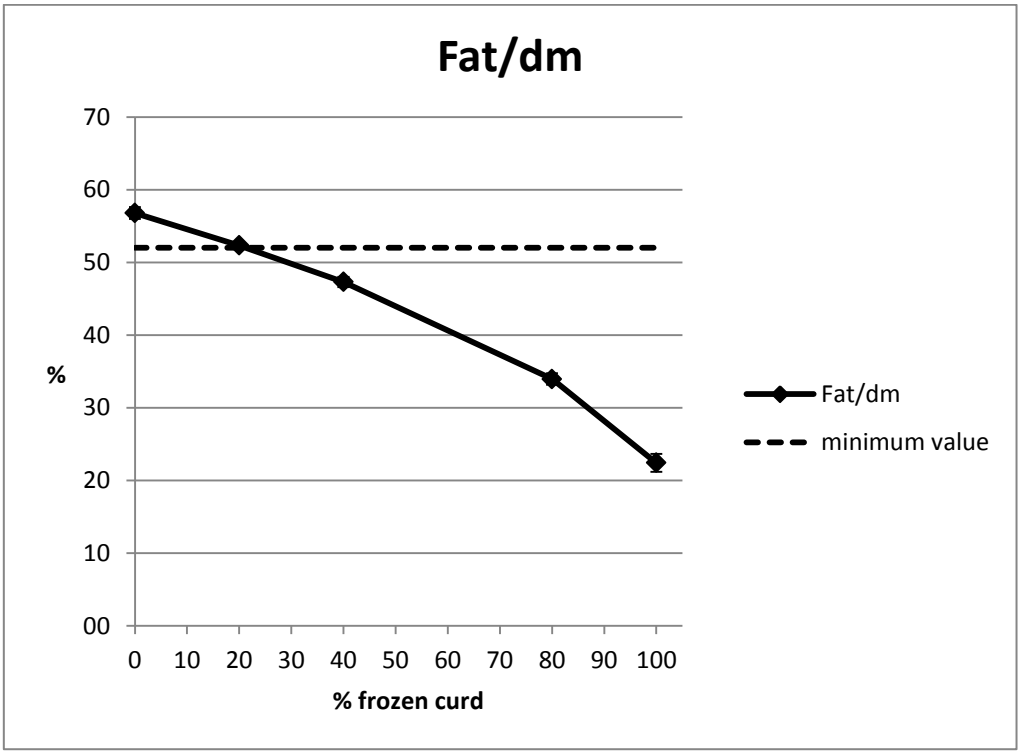


FIGURE 25 FAT/DM IN MOZZARELLA CHEESE AT INCREASING ADDITION OF FROZEN CURD

The freezing is reported in scientific literature as able to influence the salt equilibrium of the food.

Addeo et al., (1992) suggested a possible transformation of calcium from its soluble form into its colloidal phase. This conversion was reported to increase with prolonging of freezing time. When freezing was fast, thawed milk presented the same chemical composition and the same micellar structure of fresh milk. Therefore, no differences in coagulation parameters were observed in fresh and frozen milk.

In our study, the effects of frozen curd addition in mozzarella cheese on calcium content were evaluated.

The total, soluble and colloidal calcium detected in fresh buffalo mozzarella cheese and buffalo mozzarella cheese produced with frozen curd has been reported in tab. 18.

TABLE 18 TOTAL, SOLUBLE AND COLLOIDAL CALCIUM IN BUFFALO MOZZARELLA CHEESE FROM FRESH AND FROZEN CURD

	Ca		
frozen curd addition (%)	Total	Soluble	Colloidal
0	$0.250^{b} \pm 0.007$	$0.050^{b} \pm 0.004$	$0.200^{b} \pm 0.005$
20	$0.260^{b} \pm 0.009$	$0.053^{b} \pm 0.002$	$0.206^{b} \pm 0.007$
40	$0.254^{b} \pm 0.002$	$0.052^{b} \pm 0.003$	$0.202^{b} \pm 0.003$
80	$0.276^{ab} \pm 0.003$	$0.055^{ab} \pm 0.001$	$0.221^{b} \pm 0.009$
100	$0.294^{a} \pm 0.012$	$0.059^{a} \pm 0.004$	$0.235^{a} \pm 0.016$

a-b: different letters for each parameter correspond to significant differences (p<0.05)

In our experimental condition, no significant differences were detected in total, soluble and colloidal calcium in mozzarella cheese from 0 to 80% of frozen curd addition.

7.6 Furosine content

The Italian law fixes a maximum content of furosine in mozzarella cheese produced from cow and buffalo milk. This value is 12 mg/100 g of protein (D.M. 15 dicembre 2000).

Resmini et al. (1992) reported that furosine increases of 1 mg/month during storage of milk in freezing condition. Therefore the possibility to trace the presence of frozen curd through detection of furosine was investigated.

The furosine concentration of fresh mozzarella cheese and mozzarella cheese from frozen curd addition are reported in fig.26.

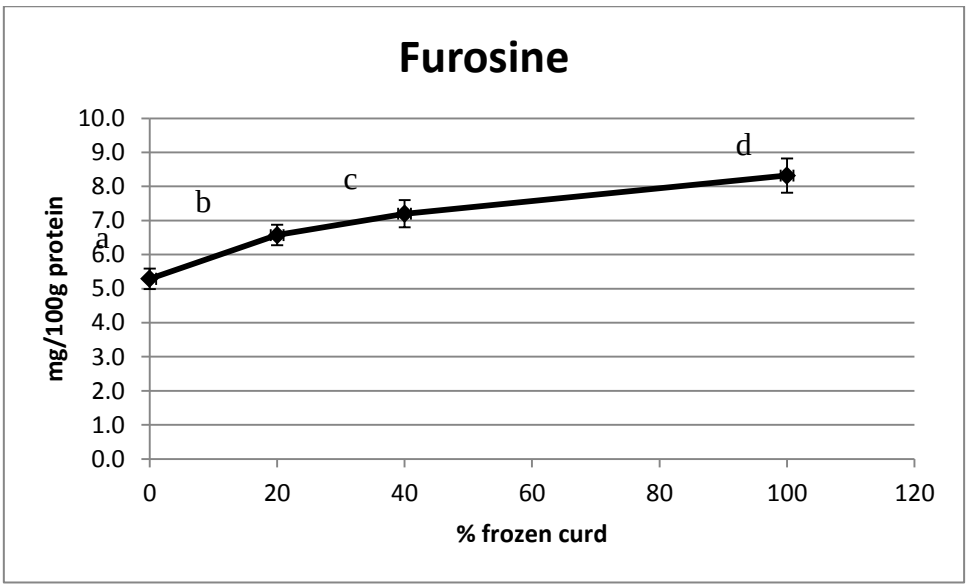


FIGURE 26 FUROSINE CONTENT (mg/100gprotein) OF MOZZARELLA CHEESE AT DIFFERENT PERCENTAGE OF FROZEN CURD

Although a growth of furosine was detected at increasing percentage of frozen curd, the legal limit of furosine in mozzarella cheese (12 mg/100 g of protein) was never exceeded. In fact, concentrations in the range 5.3-8.3 were observed.

7.7 FFA

Fat hydrolysis was evaluated at increasing percentage of frozen curd in mozzarella cheese. The results of free fatty acids are reported in tab.19.

TABLE 19 FFA IN BUFFALO MOZZARELLA CHEESE AT INCREASING PERCENTAGE OF FROZEN CURD (MEAN VALUES AND STANDARD DEVIATIONS)

% frozen curd	FFA (%)
0	0.73^{a} ±0.01
20	0.80^{b} ±0.01
40	0.80^{b} ±0.01
80	0.80^{b} ±0.01
100	0.79^{b} ±0.01

a-b: different letters correspond to statistical significant differences ($p<0.05$)

Fresh mozzarella cheese showed the lowest FFA value, which increased when frozen curd was added in cheese production. No significant differences were observed for addition from 20 to 100%.

7.8 Thermal profile analysis

The thermotropic properties of anhydrous fat and milk fat globule membrane (MFGM) extracted from fresh mozzarella cheese and mozzarella produced with frozen curd addition were studied.

MFGM thermotropic properties

The study of membrane lipids thermotropic properties can be useful to determine changes in the microenvironment of membrane lipids (Zhu and Damodaran, 2011)

The MFGM, coating the natural milk fat globules, consists of protein, glycoprotein, enzymes, phospholipids, triacylglycerols, cholesterol, glycolipids, and other minor components. MFGM can be markedly affected by treatments, such as cooling, heating, and homogenization of dairy products (Ye et al., 2002).

Zhu and Damodaran (2011) studied changes in the microenvironment of MFGM by thermotropic properties of membrane lipids, isolating the MFGM from cheese whey. According to their results, the choice of drying method can affect the morphological characteristics and the melting temperature of phospholipids in MFGM. They reported a 10°C shift in the phase transition of MFGM lipids due to changes in the microstructure of membrane lipids. Although the nature of that microstructural change was unclear, it was ascribed to many factors, such as altered protein-lipid interactions, changes in the packaging order of lipids, and/or changes in the lateral organization/distribution of the lipids.

The enthalpy values (ΔH) obtained for MFGM extracted from:

-fresh mozzarella cheese (produced from fresh curd, according to PDO specification)
-mozzarella cheese produced from 20% frozen curd mixed with fresh curd
- mozzarella cheese produced from 40% frozen curd mixed with fresh curd
-mozzarella cheese produced from 100% frozen curd

are reported in tab. 20.

TABLE 20 MELTING VALUES OF MOZZARELLA CHEESE OBTAINED FROM FRESH AND FROZEN CURD (MEAN VALUES ± STANDARD DEVIATIONS)

	peak 1		peak 2		
	Tp (°C)	**ΔH (J/g)**	**Tp (°C)**	**ΔH (J/g)**	**ΔH (J/g) Tot.**
MFGM 0%	13.54 ± 0.05	51.80^{a} ± 3.58	28.54^{a} ± 0.55	9.20^{a} ± 0.51	**61.04^{a} ± 4.09**
MFGM 20%	14.95 ± 0.25	48.13^{a} ± 0.50	29.09^{a} ± 0.13	7.11^{a} ± 0.41	**55.23^{a} ± 0.90**
MFGM 40%	14.0.1 ± 0.36	45.01^{ab} ± 0.55	30.01^{b} ± 0.24	8.40^{a} ± 0.68	**53.40^{a} ± 0.70**
MFGM 100%	13.37 ± 0.85	44.69^{b} ± 3.51	30.96^{c} ± 0.18	9.95^{a} ± 0.89	**54.64^{a} ± 4.39**

a-b: Different letters in the same column correspond to statistical significant differences ($p<0.05$)

DSC thermograms exhibited two peaks: peak 1 between 10 and 15°C and peak 2 between 7 and 10°C. Significant increase in melting temperature was observed in melting temperature of the second peak, while no significant differences were observed for the first one.

As regard enthalpy, no significant differences were observed for the peak 1 of MFGM extracted from fresh cheese whey and from whey of cheese produced with 40% frozen curd, while significant differences where observed when mozzarella was prepared with 100% frozen curd. In particular, a reduction of enthalpy was detected at increasing percentage of frozen curd. For the peak 2, no significant differences were found in the enthalpy values with increase in frozen curd.

Mozzarella cheese fat thermotropic properties

DSC melting thermograms obtained for fat extracted from fresh mozzarella cheese, mozzarella produced with 20 to 100% frozen curd are reported in fig. 27.

The thermograms of fresh mozzarella exhibited three clear peaks at about 5, 14 and 32°C

The melting below 30°C is associated to triglycerides with low molecular weight and unsaturated triglycerides with high molecular weight (Tunic and Malin, 1997).

With increase in frozen curd in mozzarella cheese, a dissolving of the peak at 5°C (highlighted) can be observed. This peak was clearly visible in fresh mozzarella, while with addition of 20% frozen curd, different profiles were obtained: one similar to fresh mozzarella cheese (but having a less marked peak) and one similar to 40 and 100% frozen curd addition. Moreover a reduction of melting temperature can be observed with increase in frozen curd in mozzarella cheese.

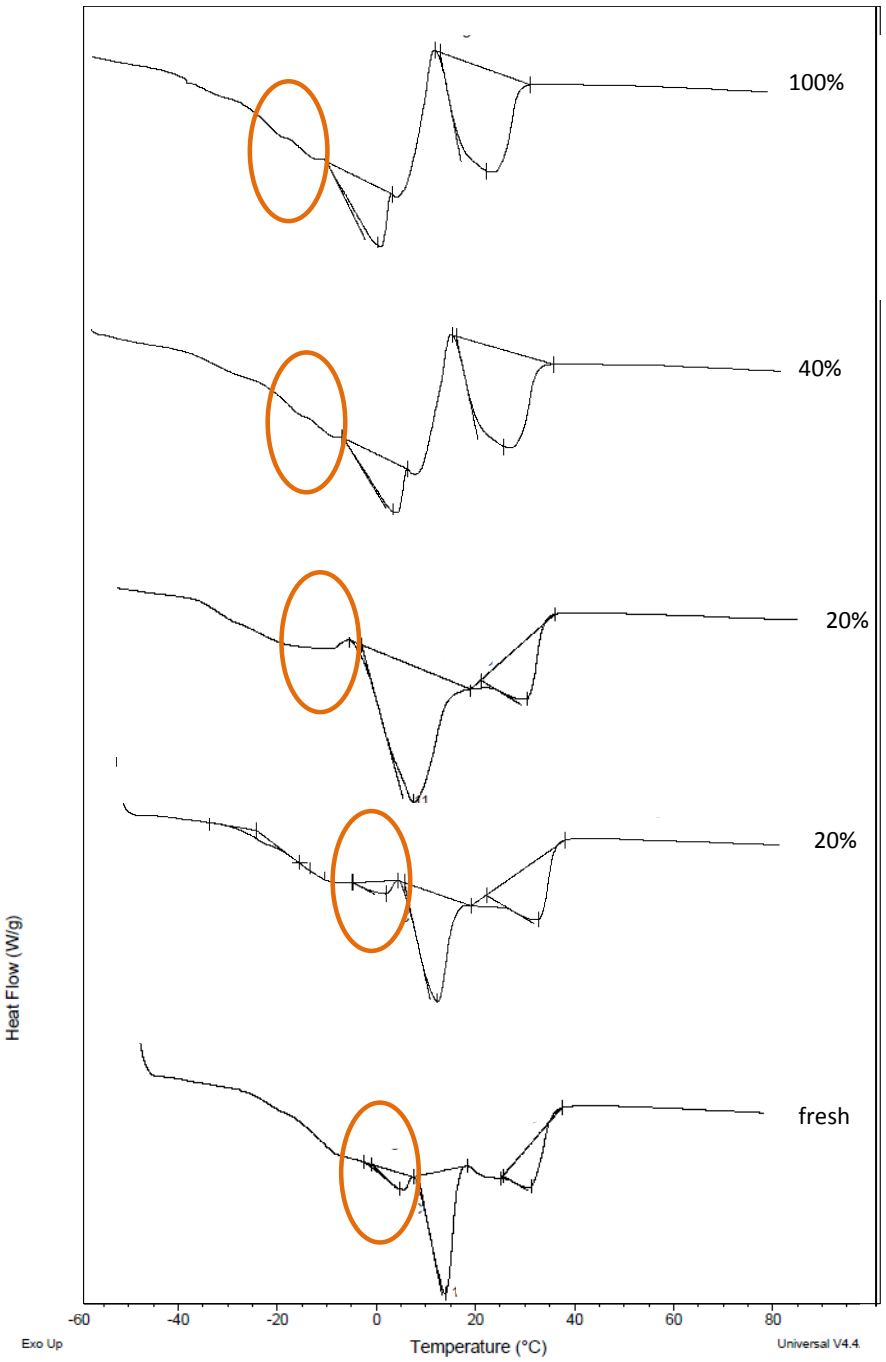


FIGURE 27 DIFFERENTIAL SCANNING CALORIMETRIC MELTING THERMOGRAMS OF FAT EXTRACTED BY FRESH MOZZARELLA CHEESE, MOZZARELLA CHEESE PRODUCED WITH 20 TO 100% FROZEN CURD

In the fig. 28, the melting peak temperatures of the three identified peaks were reported at increasing percentage in frozen curd addition.

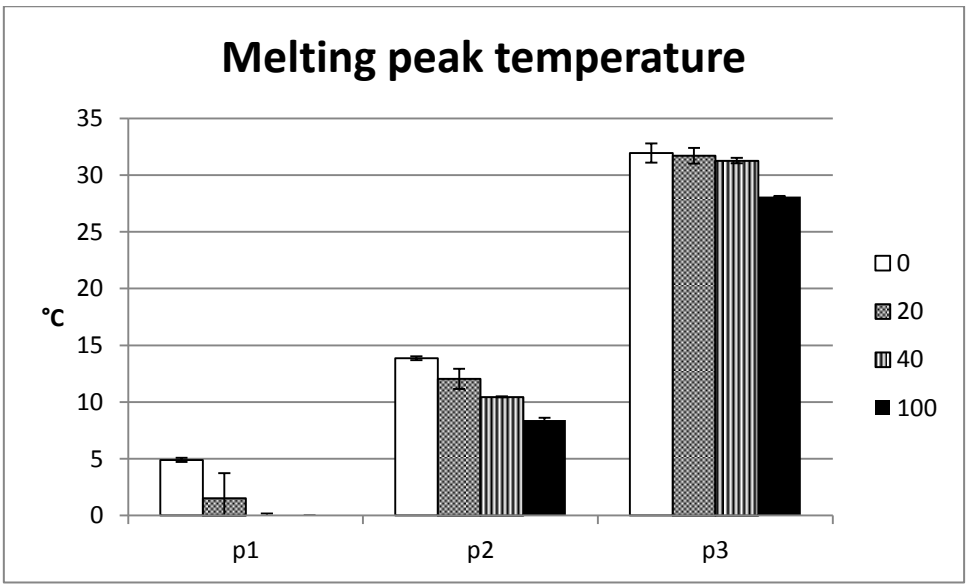


FIGURE 28 MELTING PEAKS (p1, p2 AND p3) TEMPERATURE AT INCREASING PERCENTAGE OF FROZEN CURD

The first peak showed a shift of 3.36 °C from fresh (0% frozen curd) to 20% frozen curd addition.

The second peak showed a shift of 1.82 °C from fresh to 20% frozen curd addition and further decreases of 1.59 °C and 2.03 °C when 20 and 40% respectively of frozen curd were added. A total decrease in peak 2 melting temperature of 5.44 was detected from fresh to 100% frozen curd.

The third peak showed no significant differences from fresh to 40% frozen curd, even if a decrease in temperature was observed, while a significant decrease was detected when mozzarella was prepared with 100% frozen curd. The peak 3 showed a total shift of 3.83 °C.

The reasons of the different thermal properties can be attributed to fat lipolysis. Lopez et al. (2006) reported a decrease in final melting temperature with increase in FFA during ripening of Emmental cheese. In our case, an increase in FFA of 9.5% was observed when frozen curd was added.

The crystallization profiles of the samples is reported in fig.29.

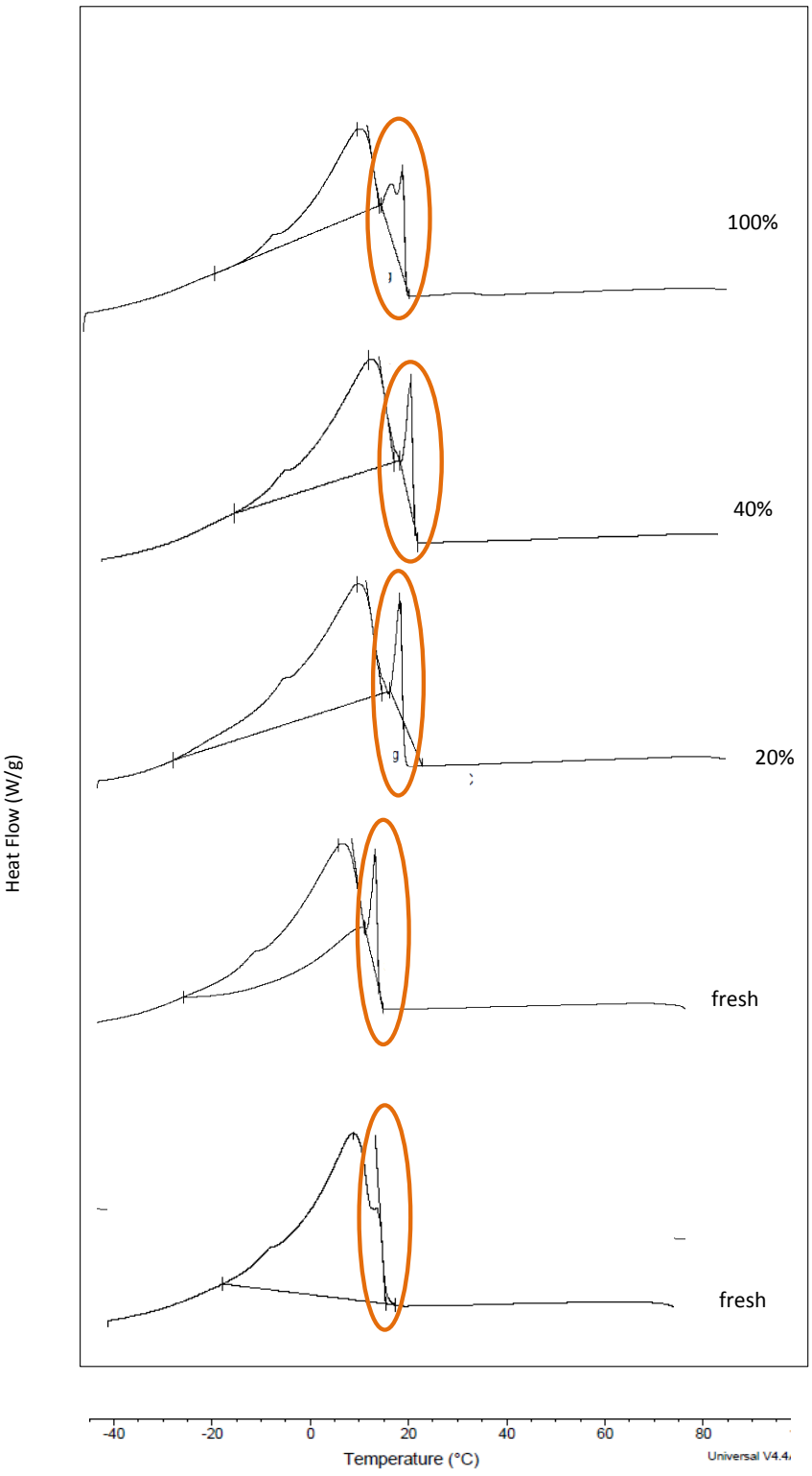


FIGURE 29 DIFFERENTIAL SCANNING CALORIMETRIC CRYSTALLIZATION THERMOGRAMS OF FAT EXTRACTED BY FRESH MOZZARELLA CHEESE, MOZZARELLA CHEESE PRODUCED WITH 20 TO 100% FROZEN CURD

The fresh mozzarella showed two different profiles: one showed the presence of only one peak, in the range of lower temperature, the second showed the presence of a further peak at higher crystallization temperatures. The area of this peak (highlighted) was found to increase with addition of frozen curd until presenting a shoulder when 100% frozen curd was added.

No significant differences were observed in crystallization temperatures of the peaks. Average values of 5.4°C and 13.4°C were detected for the first and the second peak, respectively.

7.9 Structural properties of Buffalo Mozzarella Cheese from frozen curd

Texture, X Ray and NMR analysis were performed to examine the differences between fresh mozzarella cheese and mozzarella cheese produced with frozen curd at different percentage addition.

Texture analysis

Cheese is made from curd by coagulating the casein in milk. The type of curd that develops depends upon the handling techniques, moisture content and aging durations. The characteristics of cheese can vary in texture, flavor and consistency depending on the manufacturing process. Consequently, cheese can be semi-soft, grainy, hard, smooth, crumbly or creamy. Hard cheese, for instance, contains a lower moisture and fat content.

The results of cutting test were reported as positive area under the peak, as showed in fig. 30-31 for fresh mozzarella cheese and mozzarella cheese produced with 100% frozen curd respectively.

The hardness was measured as the total positive area under the curve. This measurement records the total 'work' involved in performing the cutting test.

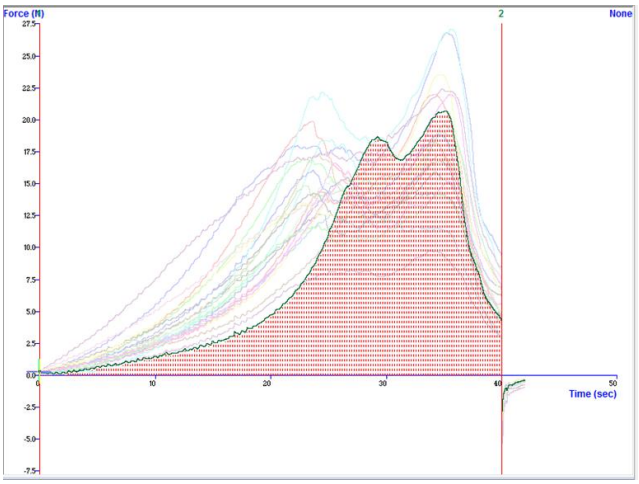


FIGURE 30 POSITIVE AREA OF FRESH MOZZARELLA CHEESE (CORE SLICE)

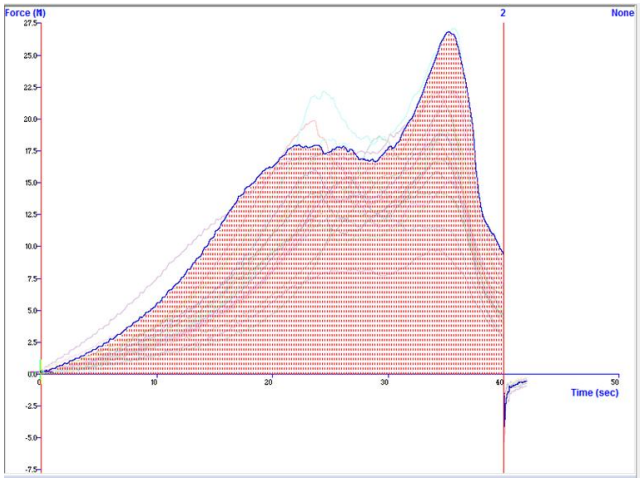


FIGURE 31 POSITIVE AREA OF MOZZARELLA CHEESE FROM 100% FROZEN CURD (CORE SLICE)

Core slices, sampled in the central part of mozzarella cheese, and lateral slices were subjected to the analysis. The results are reported in fig.32.

Higher area values indicated higher amount of energy involved in performing the test, therefore it means harder samples.

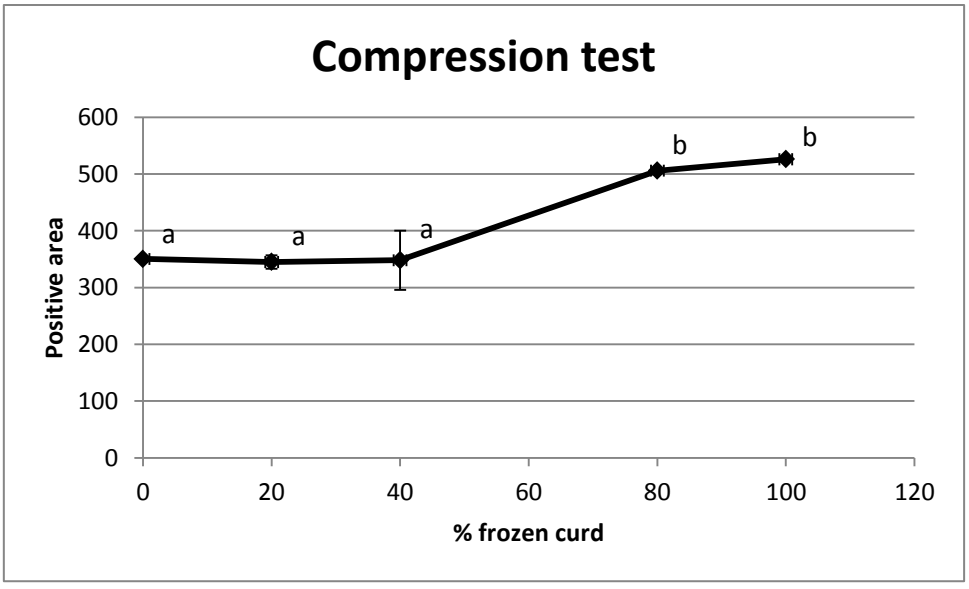


FIGURE 32 CORE SLICES OF MOZZARELLA CHEESE SAMPLES

a-b: different letters correspond to significant differences ($p<0.05$)

In the analysis of core slices, significant differences were observed for addition of frozen curd above 40%. Samples with 80 and 100% frozen curd resulted harder than fresh mozzarella. No significant difference was detected between fresh mozzarella cheese and mozzarella produced with 40% frozen curd.

The analysis was repeated many times on lateral slices of the Mozzarella cheese. In the fig. 33 obtained results were reported.

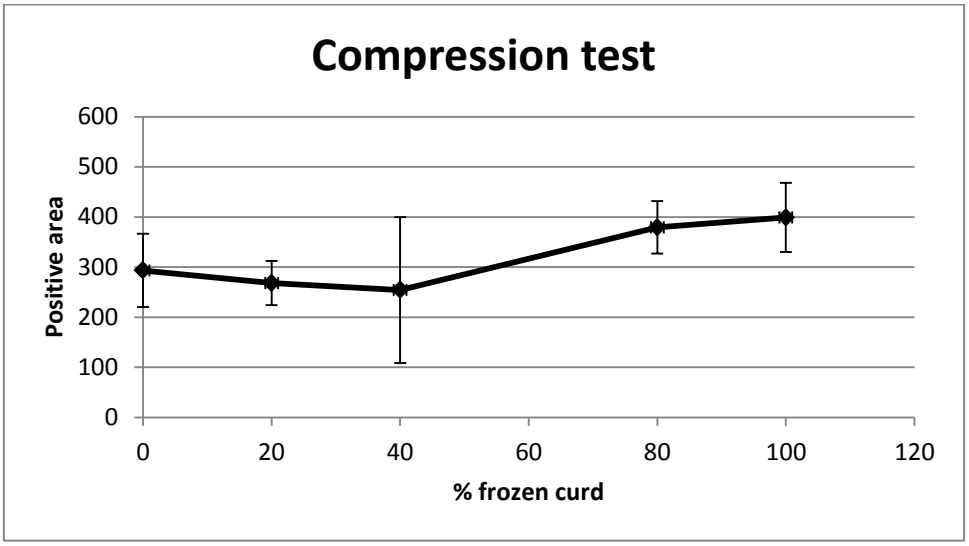


FIGURE 33 LATERAL SLICES OF MOZZARELLA CHEESE SAMPLES

($p>0.05$)

The analysis of lateral side slices of mozzarella samples resulted inadequate to detect frozen curd in mozzarella cheese. No significant differences were detected between fresh mozzarella cheese and mozzarella produced with frozen curd. This could be explained by the fact that the external slices were more susceptible than the core of the cheese to absorb water from the liquid where the cheese was stored. The process of water absorption can also occur in a inhomogeneous way. This could be responsible for the high standard deviation values detected.

X ray

X-ray mCT was used as a non-destructive and non-invasive technique to investigate the 3D microstructure of Mozzarella cheese produced from fresh and frozen curd.

The ability to measure and visualize food microstructure in 3D is important to understand how food properties are in association with processing conditions (Pinzer et al., 2012).

Food microstructure affects the physical, sensory and textural properties of products (Schoeman et al., 2016).

X-ray microtomography has found potential applications in food quality evaluation. The technique allows visualization and analysis of the architecture of the materials without sample preparation (Maire et al. 2003; van Dalen et al. 2003; Lim and

Barigou 2004). X-rays are short wave radiations, which can penetrate through the tissue. The level of transmission of these rays depends mainly on the mass density and mass absorption coefficient of the material (Maire et al. 2001; Salvo et al. 2003).

The dairy industry has been using X-ray mCT for a large number of analyses on dairy products. More recently, complex products such as cream cheese (Laverse et al., 2011) were evaluated for a variety of characteristics. Pinzer et al. (2012) used X-ray mCT to track the microstructural evolution during temperature variation in ice cream by means of time-lapse studies. The microstructure of milk powders, both loose-packed and compacted as well as spray-dried skimmed and whole milk powders was examined by Chawanji et al. (2012). This allowed the quantification of the proportion of interstitial and occluded air voids. Furthermore, the microstructural details such as the shape and size of the particles and internal voids could be characterized.

Micrographs of buffalo mozzarella cheese samples obtained by MicroCT tomography were analysed to assess the internal structure in terms of porosity.

Samples were dried to eliminate water interferences. In the fig. 34 the x Ray images of dried slices of mozzarella cheese are shown. A more compact structure was obtained when frozen curd was added. As can be observed, fresh mozzarella cheese was characterized by higher presence of voids.

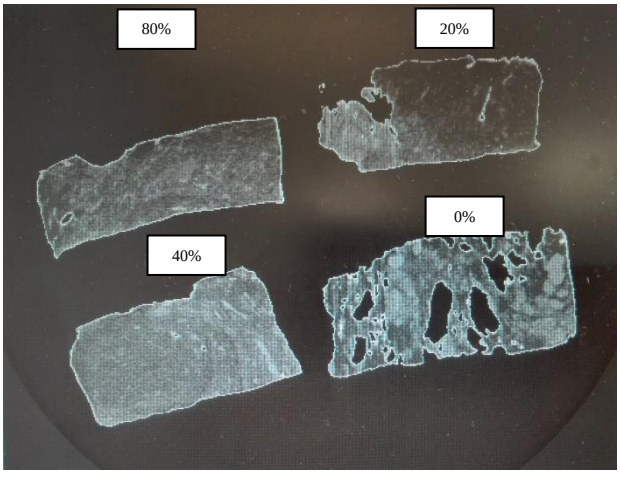


FIGURE 34 XRAY IMAGES OF CORE SLICES AT INCREASE IN FROZEN CURD ADDITION: 0% (FRESH MOZZARELLA), TO 80% FROZEN CURD

The voids were analysed to calculate their dimensions.

Different voids were defined as different regions, as indicated in fig. 35.

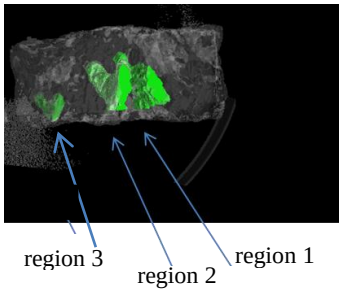


FIGURE 35 VOIDS REGIONS DETECTED IN FRESH MOZZARELLA CHEESE

The results of voids analysis are reported in tab. 21. An average value of the measures of all detected regions was reported for each sample.

One of the benefits of Xray mCT is that image analyses is not restricted to one individual slice at a time, but covers the volume in all three dimensions.

Volume data contains an incessant set of voxels that are organized in a 3D grid structure. Voxels are volumetric pixels and thus the 3D equivalent of pixels. The information from several 2D slices were merged to create a 3D image that allowed volumetric observations and measurements of the 3D microstructure. In fact, in contrast to conventional microscopy techniques, X-ray mCT allow to provide both 2D and 3D images of the whole sample (Schoulder., 2016).

TABLE 21 VOIDS DIMENSION ANALYSIS

% frozen curd	dimensions (voxel)			dimensions (mm)		
0	142	169	159	7.87	9.37	8.83
20	85	103	96	4.64	6.21	5.39
40	13	10	8	0.75	0.59	0.47
80	65	69	51	2.74	3.98	2.59
100	nd	nd	nd	nd	nd	nd

Decreasing dimensions of voids were observed with increase in frozen curd addition.

Each voxel signifies a particular area of the sample where the grey value offers information on the density properties in this region (Schoulder., 2016).

The Grey level histogram obtained for core slices images is reported in fig.36.

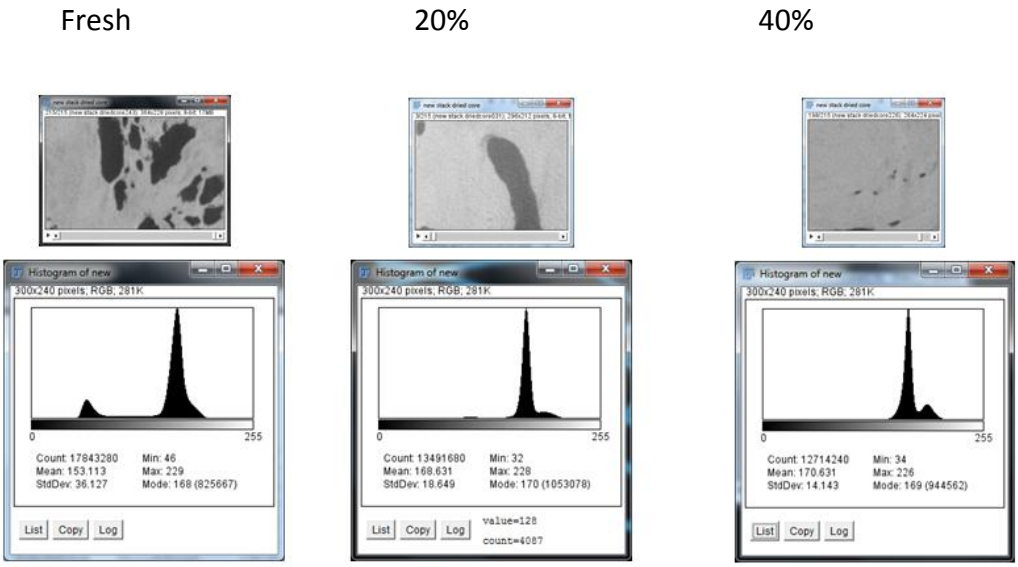


FIGURE 36 GREY LEVEL HISTOGRAM OF FRESH MOZZARELLA CHEESE AND MOZZARELLA FROM 20 AND 40% FROZEN CURD

In the image analysis, dark areas (lower grey value) represent internal voids and background, while brighter areas (higher grey value) indicate solid material (Schoeman et al., 2016). In our case, since the histograms were built taking only the central part of the images, the background was excluded and the dark areas only represented internal voids in the center of the sample slice.

As can be noted by histograms, at increase in frozen curd in Mozzarella, the first peak (representative of dark areas) decreased, with increase of a peak in the brighter area (solid material), clearly visible with addition of 20% frozen curd.

The rendering of images allowed to obtain the 3D vision of the samples (fig. 37)

As can be observed, a different internal structure was obtained at increase in frozen curd addition (0-20-40%). Voids, characteristic of fresh mozzarella cheese resulted well distributed in the sample 1.

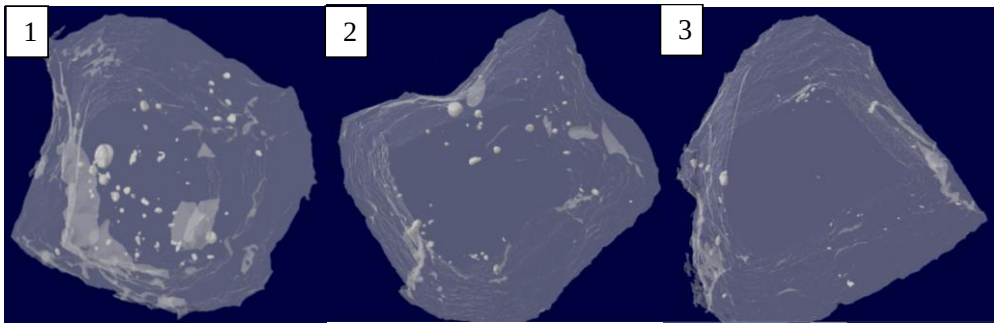


FIGURE 37 MICRO CT IMAGES (3D RENDERING) OF: 1_FRESH BUFFALO MOZZARELLA CHEESE (0%FROZEN CURD); 2_BUFFALO MOZZARELLA CHEESE FROM 20% FROZEN CURD; 3_BUFFALO MOZZARELLA CHEESE FROM 40% FROZEN CURD

Since the core of the sample could be not representative of the entire structure of mozzarella cheese, the x Ray analysis was also performed on the whole Mozzarella cheese. The samples were freeze dried to eliminate water.

The results of voids analysis found in the whole mozzarella cheeses are reported in tab.22.

TABLE 22 VOIDS ANALYSIS IN FREEZE DRIED SAMPLES

	frozen curd (%)					
	0		20		40	
Void regions	Diameter [mm]	Volume [mm³]	Diameter [mm]	Volume [mm³]	Diameter [mm]	Volume [mm³]
1	69.51	4705.56	65.47	3398.34	60.34	2612.46
2	10.67	58.6	15.14	268.79	24.2	727.74
3	10.55	39.48	11.07	66.98	12.45	78.43
4	13.4	25.97	13.0	21.02	8.41	20.6
5	11.49	23.06	9.36	10.31	5.51	12.61
6	10.65	22.65	7.49	11.45	6.31	8.92
7	-	-	8.23	5.22	9.95	7.84

Also in this case, when the whole cheese samples were analysed, the total volume of voids resulted higher in fresh mozzarella cheese (fig. 38).

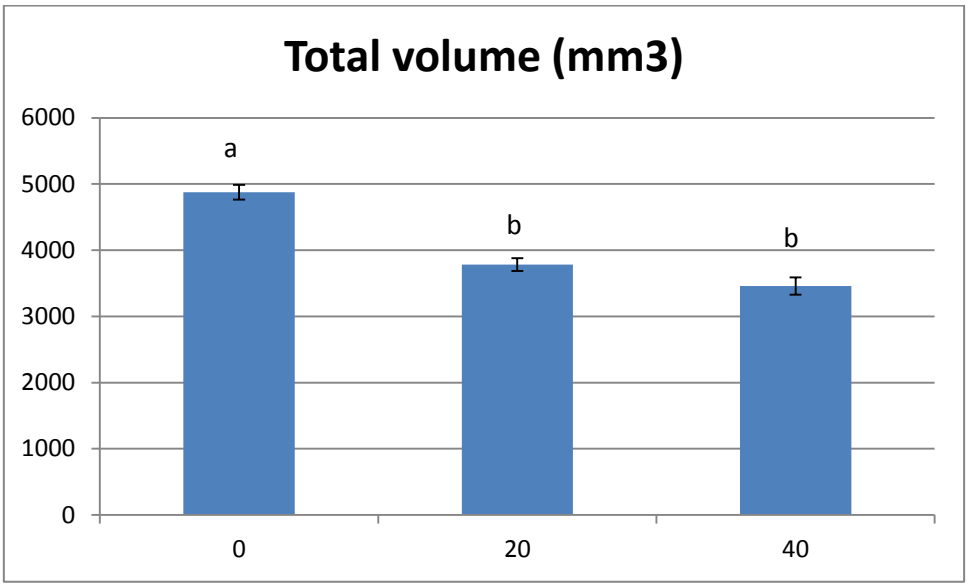


FIGURE 38 TOTAL VOLUME OF VOIDS IN FREEZE-DRIED SAMPLES

A significant decrease of total volume of voids was observed when frozen curd was added.

The overlaying of 3D images of voids in whole fresh mozzarella cheese (RED) and mozzarella produced with 40% frozen curd(BLU) is reported in fig. 39.

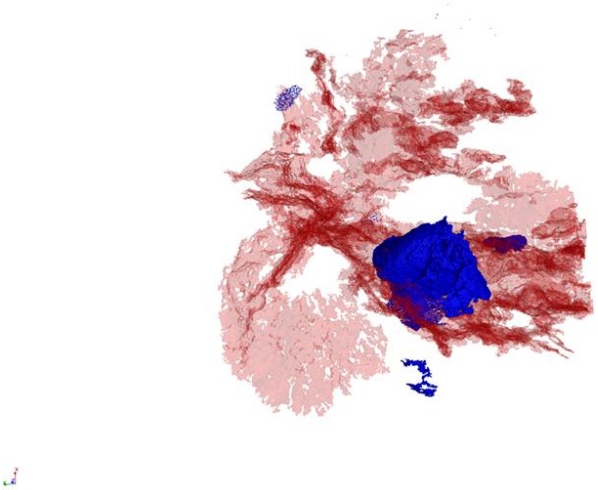


FIGURE 39 VOIDS REGIONS OVERLAYING OF FRESH BUFFALO MOZZARELLA CHEESE (RED) AND BUFFALO MOZZARELLA CHEESE PRODUCED WITH 40% FROZEN CURD

In our experimental conditions, X Ray-microCT allowed to determine differences in the texture of fresh Mozzarella cheese and Mozzarella cheese produced with frozen curd.

NMR

Low resolution NMR was used to investigate the state and the distribution of water in Mozzarella cheese. Modifications that can occur during freezing of mozzarella cheese, which detected significant differences in water relaxation time were observed by Kuo et al., 2003 after mozzarella thawing. These authors report that the formation of ice crystals may destroy bonds between water and protein fiber changing water distribution. In fact, water relaxation, is affected by food network structure as a result of water-macromolecule interactions (Gianferri et al., 2007).

Mozzarella cheese is a complex system and its water distribution differs from most other cheeses because of a particular phase of the cheese-making process which correspond to the curd stretching, responsible of changes of its microstructure. During the stretching, an elastic network of oriented parallel protein fibers is created (kuo et al., 2001).

Low resolution NMR, that has low sensitivity, is in general used to collect information on food macromolecules: water, lipids, proteins and carbohydrates. In particular, in our case, it was used to investigate the state and distribution of water in Mozzarella and to assess if water relaxation time can be suitable to discriminate between fresh mozzarella cheese and mozzarella produced from frozen curd.

The analysis was performed on more sections of the same sample. In fig. 40 the results obtained for the analysis of fresh buffalo mozzarella cheese and buffalo mozzarella cheese produced from 20% and 100% frozen curd are reported.

In black color, the profiles of the sections analysed in fresh mozzarella cheese. In red color, the profiles of the sections analysed in mozzarella from 100% frozen curd. In green color, the profiles of the sections analysed in mozzarella from 20% frozen curd.

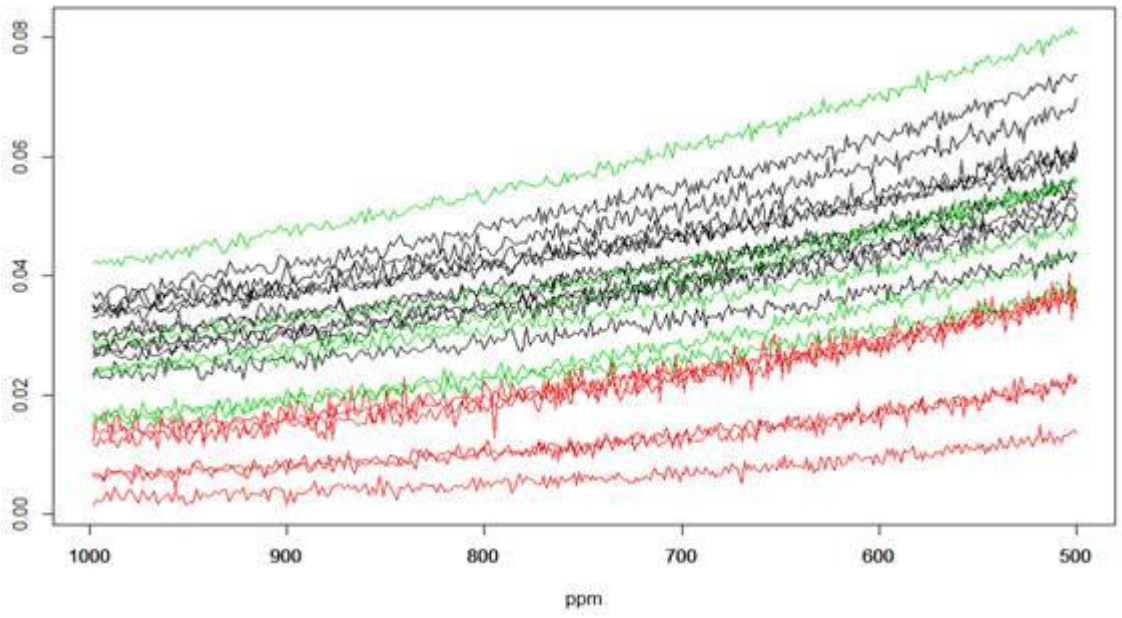


FIGURE 40 RELAXATION RECOVERY CURVES OF FRESH BUFFALO MOZZARELLA CHEESE (BLACK), BUFFALO MOZZARELLA CHEESE PRODUCED FROM 20% (GREEN) AND 100% (RED) FROZEN CURD

As expected, the mozzarella cheese relaxation curves showed an exponential behavior because of the presence of different components and different structural elements in the sample, as reported by Gianferri et al (2007).

The three different types of mozzarella showed a different position in the diagram, although Mozzarella cheese with 20% frozen curd showed some replicates in the range of fresh mozzarella cheese relaxation times. This could be due to similarities in water distribution between fresh and mozzarella from 20% frozen curd.

7.10 H-NMR metabolomic analysis to assess traceability of frozen curd in buffalo mozzarella PDO cheese

High resolution NMR was employed to determine the 'chemical fingerprint' of the cheese as affected by addition of frozen curd. This technique is used, in fact, to identify also unknown and unexpected substances in food (Gianferri et al., 2007).

The H-NMR profiles of water soluble metabolites in fresh Buffalo Mozzarella PDO cheese (black) and Mozzarella prepared from 20 and 100% frozen curd (in green and red colours respectively) are shown in fig. 41.

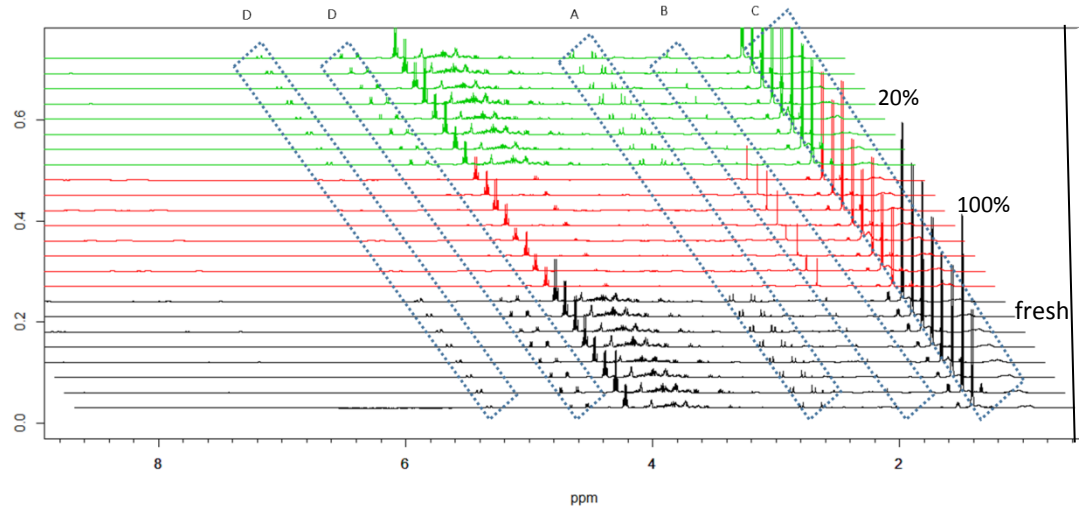


FIGURE 41 H-NMR SPECTRA OF FRESH MOZZARELLA CHEESE AND MOZZARELLA PREPARED FROM 20 AND 100% FROZEN CURD. A) CITRATE, B) ACETATE, C) LACTATE AND D) DISACCHARIDES

The main signals were assigned to the major organic acids (citric, acetic and lactic), carbohydrates, minor amino acids, and other components, clearly visible in the magnification of a single spectra obtained for mozzarella cheese and reported in fig. 42.

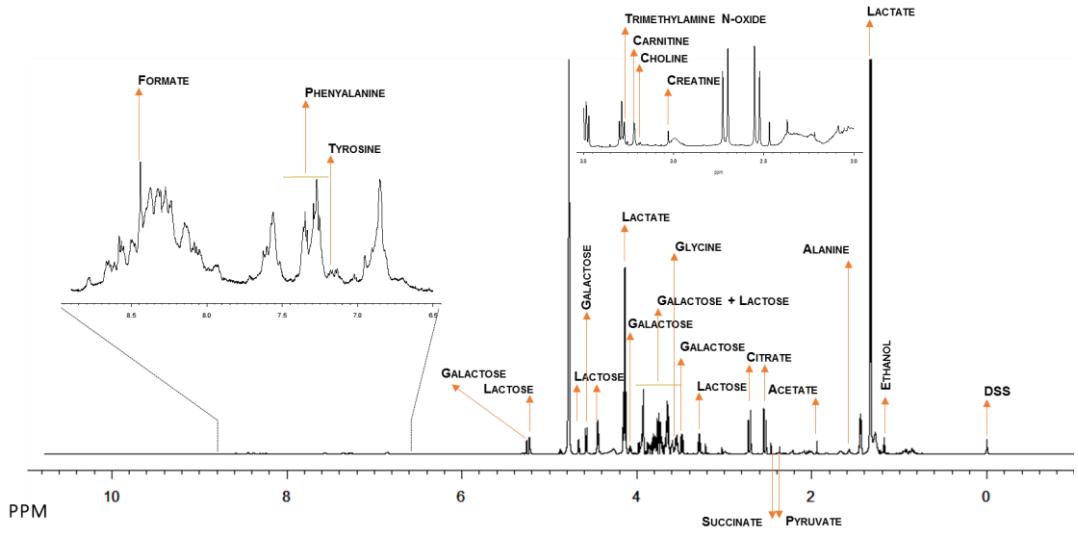


FIGURE 42 H-NMR SPECTRUM OF AN AQUEOUS EXTRACT FROM BUFFALO MOZZARELLA CHEESE

As expected, the NMR spectrum revealed the predominance of lactate resonance signals. Lactate was the most abundant component because it is the main product of lactose fermentation during cheese making. In addition, several signals from many metabolites were detectable.

The high-field region (0.0–3.0 ppm) of the NMR spectrum showed the resonance signals arising from aliphatic groups of organic acids belonging to the lactose and galactose glycolytic pathways and Krebs (or citric acid) cycle, as well as from alcohols. In particular, the signals arising from ethanol, lactate, acetate and citrate were clearly identified. In Buffalo Mozzarella cheese, citrate and acetate are involved in the Krebs cycle where they act both as a substrate and a product. Ethanol arises, instead, from fermentation process (Gianferri et al., 2007).

In the mid-field region (3.0–5.5 ppm) of the proton spectrum, the main contributions arose from the two principal carbohydrates of Mozzarella cheese (lactose and galactose) and overlapped contributions from amino acids derivatives, and organic acids (principally lactate).

The signals in the low-field region (5.5–10.0 ppm) were the weakest in the spectrum and arose from the aromatic groups of amino acids tyrosine (Tyr) and phenylalanine (Phe). Other regions in the low field region remained unassigned and probably attributable to phenols (Belton et al., 1997 and Gil et al., 2000).

The metabolic data collected for fresh mozzarella cheese, mozzarella cheese prepared with 20% frozen curd and mozzarella cheese prepared with 100% frozen curd were analysed by Principal Component Analysis.

The PCA score plot is reported in fig. 43.

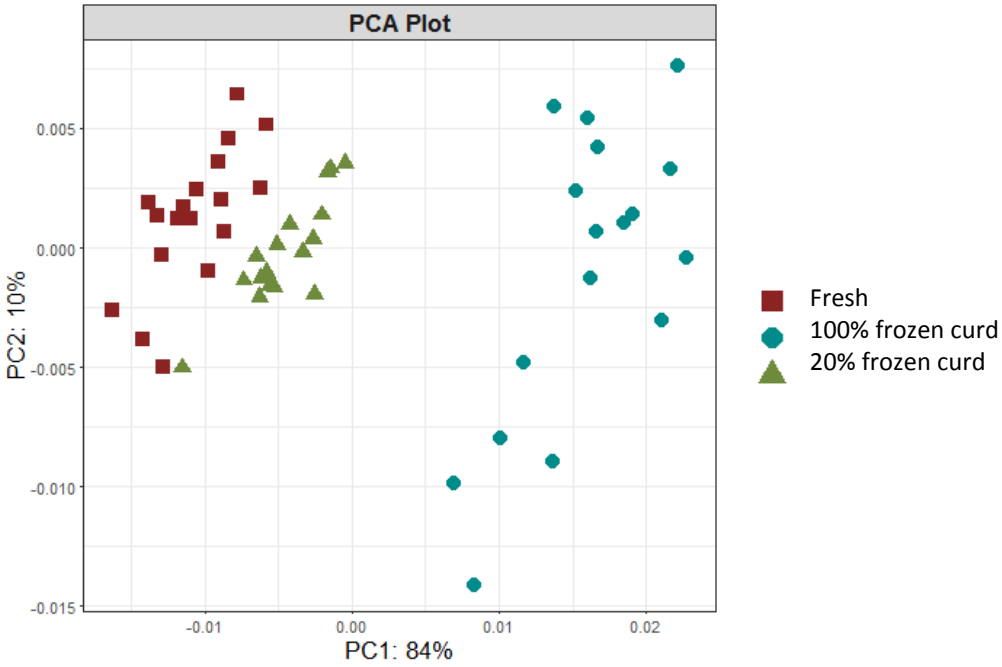


FIGURE 43 PCA SCORE PLOT OF FRESH, 20% AND 100% FROZEN CURD MOZZARELLA CHEESE

A different distribution of the samples can be observed. Mozzarella cheese made with 100% frozen curd resulted so different to explain the 84 % of variance.

In fig. 44 the PCA analysis of binned data is reported. The images clearly identified the three groups of samples: fresh, 20% and 100%. The first two principal components of PCA explained the 98% of total variance. Most of the differentiation between the three groups was detected mainly along the PC1 axis, to which 97% of variance was associated.

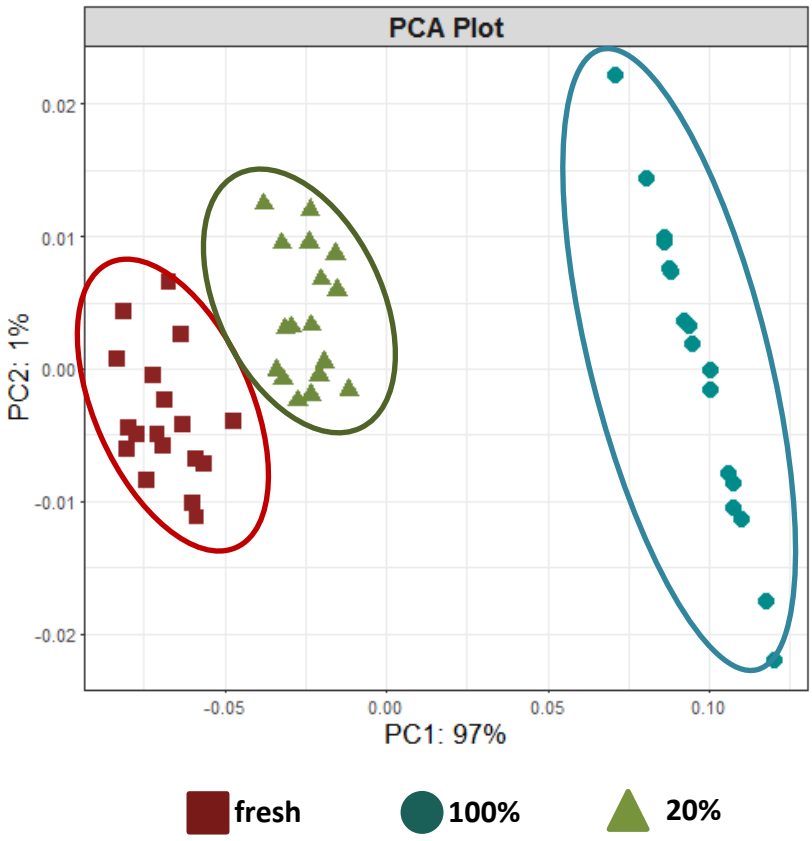


FIGURE 44 PCA SCORE PLOT (BINNED DATA) OF FRESH, 20% AND 100% FROZEN CURD MOZZARELLA CHEESE

Fresh mozzarella resulted mainly scored in the negative PC1 and PC2.
The loading plot in fig. 45 shows the metabolic compounds contributing to PC1 and PC2 variables and, therefore, responsible for the association of the samples.

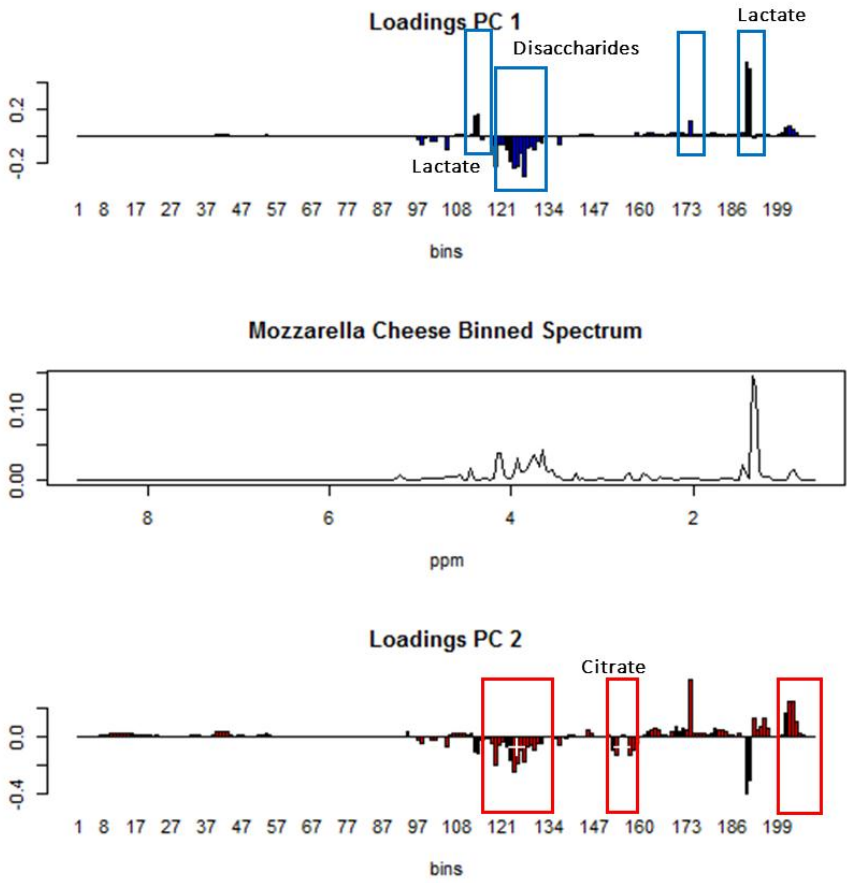


FIGURE 45 LOADING PLOT OF MOZZARELLA CHEESE BINNED SPECTRUM

The main variance was associated to PC1. As can be seen, the molecule responsible of the differentiation was the lactic acid. The values of lactic acid found in the three different groups of samples (fresh, 20% and 100% frozen curd) are reported in the in the box plot in fig. 46. H-NMR experiments were repeated after 2, 3 and 4 days of storage.

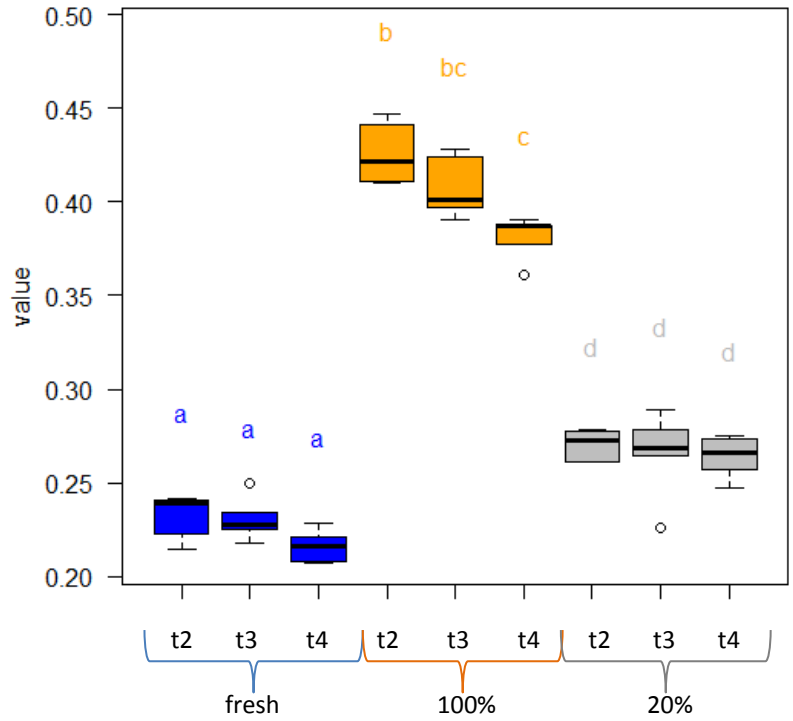


FIGURE 46 BOX PLOT OF LACTIC ACID IN MOZZARELLA CHEESE AT DIFFERENT TIMES OF STORAGE (2,3 AND 4 DAYS)

a-d: different letters correspond to significant differences

Lactic acid was recognized as a minor component in fresh mozzarella molecular profile.

Increase in lactate was detected when increasing percentage of frozen curd were used for mozzarella production. No significant differences were detected during preservation of fresh mozzarella cheese and mozzarella cheese from 20% frozen curd, while a significant decrease was obtained in mozzarella cheese with 100% frozen curd.

The increase in lactic acid could be due to microbial and biochemical reasons. Mesophilic and thermophilic lactic acid bacteria are characteristics in dairy products and responsible for lactate production (Cogan et al., 1997). Then, lactic acid can derive from the main biochemical pathway for lactate production in mozzarella cheese which consist in the glycolysis of hexose monosaccharides, such as glucose or galactose, deriving from the breakage of glycosidic bonds in the lactose disaccharide (Mazzei and Piccolo, 2012).

7.11 Protein and peptide profiles of Mozzarella cheese soluble fraction

MALDI TOF technique was used to study the proteins and peptide pattern of Buffalo Mozzarella cheese as affected by presence of frozen curd.

In fig. 47 the spectra of the proteins extracted form Mozzarella samples made with (a) 100% fresh curd, (b) 20% frozen curd and (c) 100% frozen curd are compared.

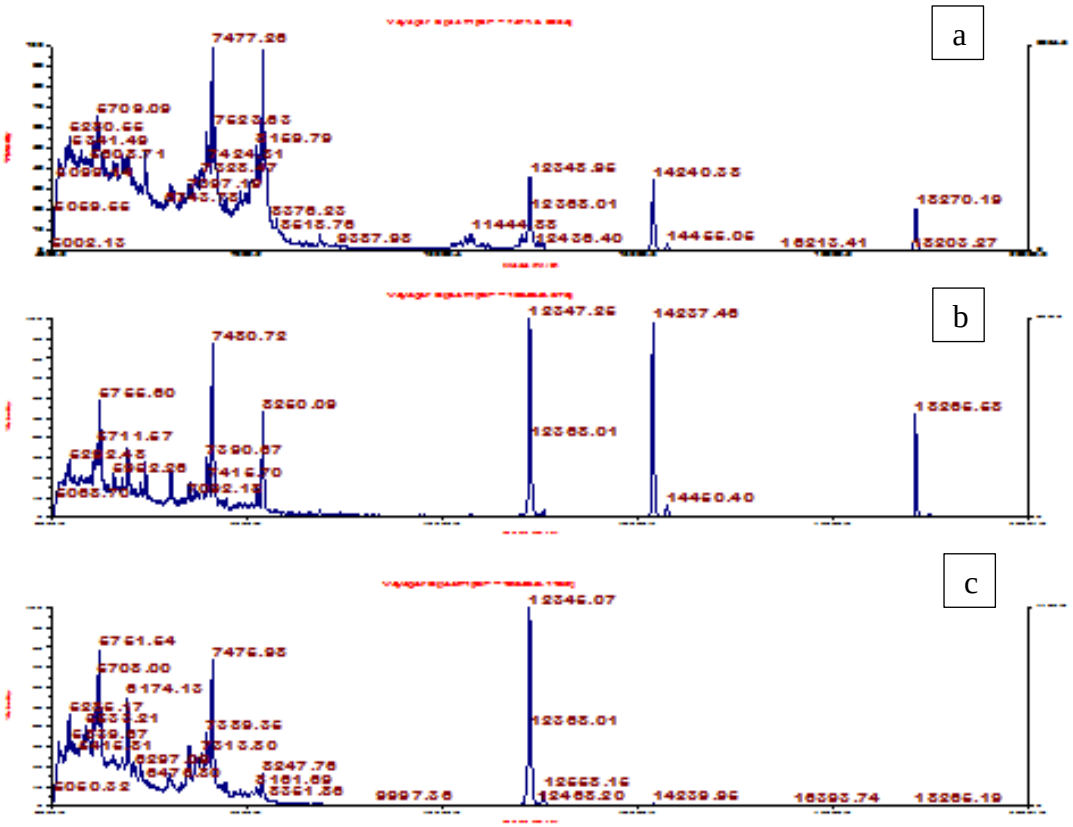


FIGURE 47 MALDI TOF MASS SPECTRA IN THE RANGE 5-20KDA OF THE PROTEINS EXTRACTED FORM MOZZARELLA SAMPLES MADE WITH (A) FRESH CURD, (B) 20% FROZEN CURD AND (C) 100% FROZEN CURD

The spectra showed several differences. In the cheese made with fresh curd, the main whey proteins aLA and bLG were observed, together with proteose-peptones and parak-casein. In the cheese made with 100% frozen curd, all these components were absent, the main protein being the para-k casein at 12kDa. In the cheese made with 20% frozen curd, the protein pattern was intermediate. These observations were confirmed by analysis of LMW peptides, in the fig.48 .

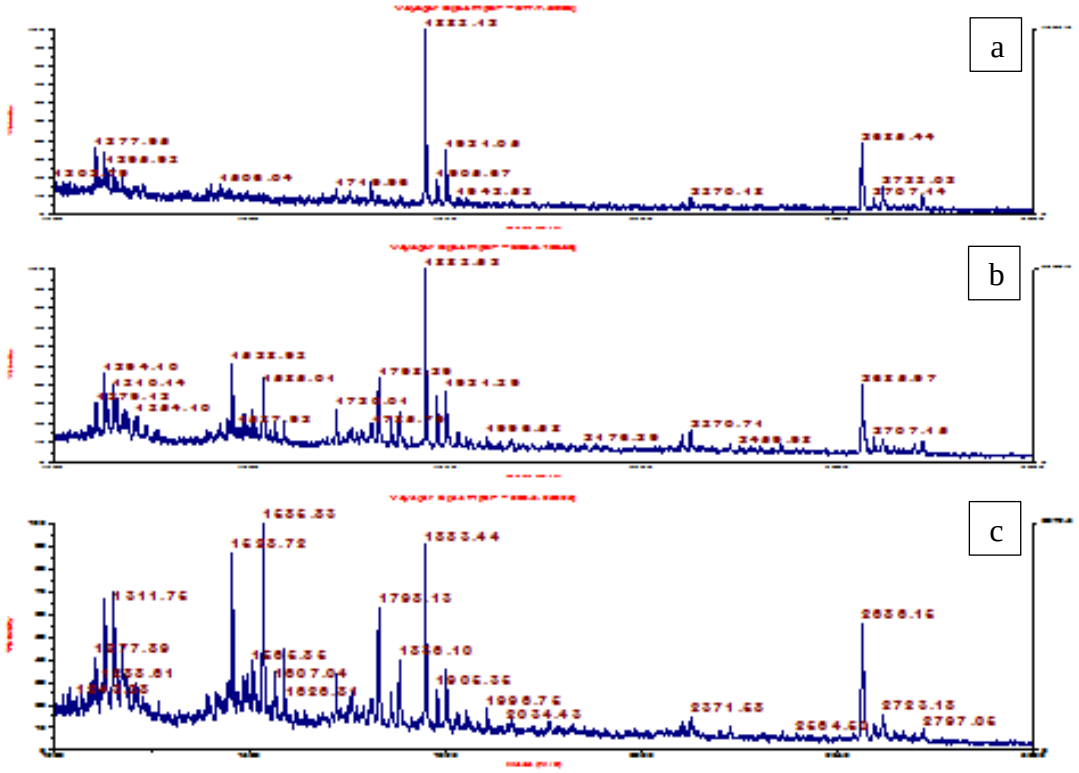


FIGURE 48 MALDI TOF MASS SPECTRA OF LMW PEPTIDES OF THE PROTEINS EXTRACTED FORM MOZZARELLA SAMPLES MADE WITH (A) FRESH CURD, (B) 20% FROZEN CURD AND (C) 100% FROZEN CURD

The peptides observed derived from the proteolysis of the main casein fractions beta and as1, already present in the milk used for curding as previously demonstrated in previous studies carried out on bovine milk. The spectra, however, showed several qualitative and quantitative differences in the peptide patterns of Mozzarella prepared with either fresh or frozen curd.

Experiments carried out with limited proteolysis using pepsin showed a very different degradation pattern. In fig.49, the protein and peptide profiles of Mozzarella cheese after simulated gastrointestinal digestion are compared.

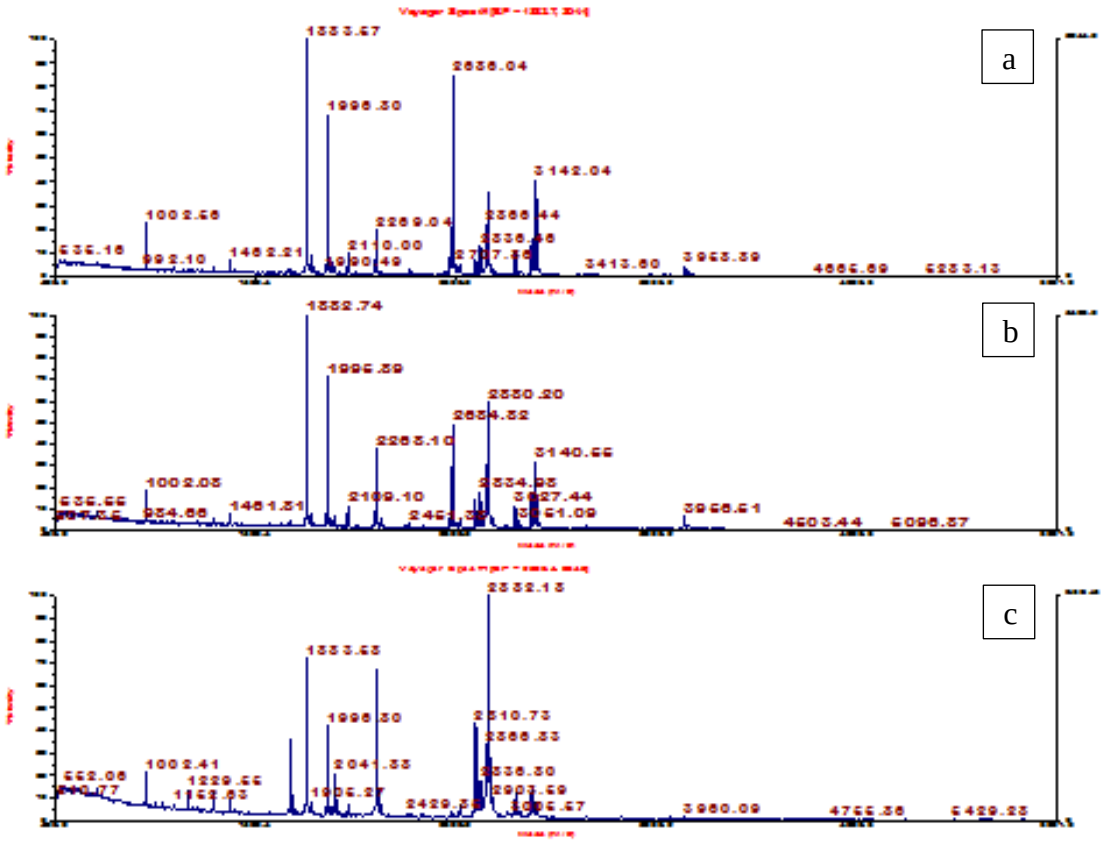


FIGURE 49 DIDASC MALDI TOF MASS SPECTRA OF PROFILES OBTAINED AFTER USING PEPSIN IN MOZZARELLA SAMPLES MADE WITH (A) FRESH CURD, (B) 20% FROZEN CURD AND (C) 100% FROZEN CURD

In spite of a general similarity, several LMW peptides marked the differences among fresh and frozen curd in cheese-making.

8. Conclusions

Campana Buffalo Mozzarella cheese is protected by PDO specification which is a guarantee of freshness for consumers. However, increased consumer demand, limited availability of buffalo milk and the increased economic profits make Mozzarella cheese a target for adulteration. Since the cheese has to be produced exclusively by using fresh buffalo milk processed within 60 hours after milking, the use of frozen milk or curd is not allowed. Food control authorities require analytical methods to discriminate between fresh Mozzarella PDO cheese and Mozzarella produced from frozen intermediates, which are not produced according to the PDO manufacturing process.

The aims of this work were to assess the chemical and structural modifications that may occur during the freezing of cheese intermediates and to evaluate Mozzarella cheese quality obtained by frozen curd.

Firstly, markers of freshness identified in scientific literature (γ4-CN and αs1-I CN) were evaluated. According to our results, γ4-CN was not effective to discriminate mozzarella cheese produced by frozen milk or curd, since keeping samples at freezing temperatures for 9 months, no significant differences were found in the γ4-CN concentration. Since no increase in γ4-CN was observed during the freezing time, false negative cases could be created. The fragment αs1-I CN was also found not efficient in evaluating conformity to PDO, since the same casein profile was observed in fresh and frozen samples. The αs1-I casein fragment was not detected in any analysed samples. It could be possible that in our case, a high quality of raw material and a controlled process avoided conditions for the development of an intense proteolysis. Proteolytic action of milk enzymes can appear more or less intense depending on many factors, such as the process conditions, animal health, phase and number of lactations. Our raw material (fresh buffalo milk) showed quality and hygiene parameters within the limits provided by the current legislation.

The possibility to trace the presence of frozen curd through detection of furosine was also investigated. Notwithstanding a significant increase was detected during freezing, the limit fixed by Italian law was never exceeded.

The study of lipids demonstrated no significant differences in fatty acid and triglycerides profiles after freezing storage. The analysis of FFA showed also no significant differences between fresh and frozen curd, while an increase in FA in mozzarella cheese was detected. Mono-diglycerides (MDG), examined as an index of lipolytic activity, showed an increase in 1,2-Dipalmitin and 1,3-Diolein during freezing. The analysis of lipid compounds by differential scanning calorimetry showed significant differences in the enthalpy of fat extracted by mature curd (a decrease of about 30%) after 9 months of storage at -20°C, which may be associated to an influence of freezing storage on fat crystallization.

The addition of frozen curd in mozzarella cheese caused a change in chemical composition. Fresh Mozzarella cheese and Mozzarella produced from 20% frozen curd showed moisture and fat/dry matter parameters within the limit provided by PDO specification.

The analysis of lipids revealed a slight increase in FFA when frozen curd was added.

Mozzarella samples with increasing percentage of frozen curd showed different melting and crystallization profiles. Moreover, a reduction of melting temperature was observed.
By a structural point of view, the increase in frozen curd addition led to a harder texture of mozzarella cheese. X-Ray analysis revealed a spongy texture of the fresh product and allowed to discriminate fresh mozzarella, characterized by higher presence of voids, from mozzarella made with frozen curd. A significant decrease was observed in the total volume of voids when frozen curd was added.
Although the results were preliminary, the analysis based on both low- and high-resolution NMR seemed to be a useful approach to detect fraudulent addition of frozen curd in Buffalo Mozzarella PDO cheese.
Differences were also found in protein and peptide profile of Mozzarella cheese soluble fraction and in peptide pattern after simulated gastrointestinal digestion. A full structural identification of cheese peptides could lead to the identifications of molecular marker effective for discriminating the different products.

Notwithstanding chemical and textural modifications were found, further studies on quality assessment of Mozzarella cheese are necessary, since many factors, such as manipulation, technological process and other reasons can influence the chemical and textural composition of this particular cheese. One of the first step would be to increase the number of samples to establish a range of reference values for fresh Buffalo Mozzarella PDO cheese. This could provide, in the future, food control authorities with analytical methods in order to trace the presence of frozen curd in Mozzarella cheese which is not produced according to the PDO specifications.

Acknowledgments

This research was partially supported by the Ministry of Health, within a project carried out in collaboration with the Istituto Zooprofilattico Sperimentale del Mezzogiorno - Portici, Italy (IZSME 02/13 RC).

It is crucial to extend gratitude to all organizations, professors and doctors who participated in the work, taking part in the analysis, giving technical assistance and supplying samples. In the alphabetic order: Dr Biondi L., Istituto Zooprofilattico Sperimentale del Mezzogiorno, Portici, Italy; Prof Ferranti P., Department of Agricultural and Food Sciences - University of Naples Federico II, Portici, Italy; Dr. Fiore A., Division of Food & Drink - Abertay University, Dundee, Scotland; Dr. Nicolai A., Department of Agricultural and Food Sciences – University of Naples Federico II, Portici, Italy; Dr Picone G., DISTAL – University of Bologna, Cesena, Italy; Dr Pizzolongo F., Department of Agricultural and Food Sciences – University of Naples Federico II, Portici, Italy; Dr. Romano A., CAISIAL - University of Naples Federico II, Portici, Italy; Dr Urbinati E., DISTAL – University of Bologna, Cesena, Italy and dairy plants Caseificio F.Lli Di Lascio (SA - Italy), Industria Casearia Cirigliana (CE – Italy) and Amodio s.r.l. (NA – Italy).
A special thanks to my tutor Prof. Raffaele Romano for his inspiring guidance and encouragement during these years. His support and advices were fundamental for the realization of this research.

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Manzo N, Biondi L, Nava D, Capuano F, Pizzolongo F, Fiore A and Romano R (2017) On The Possibility to Trace Frozen Curd in Buffalo Mozzarella Cheese. J. Food Res. 6 (1): 104-111

Peer-reviewed conference proceedings

Pizzolongo F, **Manzo N**, Pagliuca A, Romano R. Evoluzione della furosina nel trattamento termico del latte di bufala - 12° Congresso Italiano di Scienza e Tecnologia degli Alimenti, Fiera Milano, Rho, in Ricerche e innovazioni nell'industria alimentare vol. XII Editore Chiriotti (ISBN: 88-85022-96-0), 3-4 Maggio 2015.

Manzo N and Romano R. Identification of possible markers for traceability of frozen curd in mozzarella di bufala campana pdo cheese. Proceedings book XX Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, 23rd-25th September 2015, 170-171.