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Assessment of 'freshness' in bovine mozzarella cheese

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ABSTRACT

The Italian dairy industry is greatly engaged in meeting the demand of high-moisture bovine mozzarella cheese consumers, who have high expectation regarding its freshness. Unfortunately, such a feature cannot be verified since the labelling regulation only considers the indication of the expiry date, and not the day of manufacturing. The present investigation aimed at assessing the freshness of HMMC by monitoring the proteolytic changes during storage at different temperature conditions for different times using an already tested index of freshness and the quantification of α_{S1}-I-CN fragment. The study was performed on PDO samples of known origin and was then applied to evaluate the status of 58 samples of unknown origin purchased from the market. Results showed that the index of freshness and α_{S1}-I-CN quantification could be used to double-check the 'freshness' of the products, allowing the guarantee to the consumer of the quality stated on the label.

1. Introduction

'Mozzarella' is the general term used to indicate a stretched curd cheese, obtained mainly from cow or buffalo milk. The curd stretching process is the result of the application of high temperature to chemically or biologically acidified curds, which leads to a smooth and fibrous texture (Ribero, Rubiolo, & Zorrilla, 2007). High-moisture mozzarella cheese (HMMC) is one of the most popular Italian food products, it is eaten mostly as fresh and when it is manufactured from cow milk it is also named 'fiordilatte' or as 'mozzarella fiordilatte'. In recent years, this cheese has gained two EU acknowledgements as high-quality product: Traditional Specialty Guaranteed (Regulation EC No. 2527/98) and Protected Designation of Origin (Mozzarella di Gioia del Colle, Reg. UE 2020/2018 (09/12/2020) – GUUE L. 415–09/12/2020.

HMMC is highly appreciated by consumers worldwide and represents a profitable product for the dairy industry, but it is characterised by a short shelf life because of its high moisture content. One of the most important sensory attributes is 'freshness', a term related to the use of fresh milk in the manufacturing process and the preservation of the typical aroma and texture; actually, most consumers consider mozzarella as a fresh product within two days from production, then it is used for cooking. Nevertheless, the preservation of the freshness characteristics may last longer, if the quality of the raw matter is good and the manufacturing process has been correctly carried out both under the hygienic and technological point of view. In this context, one of the major problems for the consumers is the lack of the wording relating to the 'date of manufacture' of the product on the label, which could help for the evaluation of 'freshness'. This type of information is particularly important for the PDO product, whose production must adhere to strict specifications and have to be fully traced.

Actually, the demand of mozzarella cheese is increasing all around the world and the growth of the markets has led to the need of adopting a less expensive and more competitive technological solution for its preparation. Consequently, many dairy industries have shifted their goals from improving the quality and guaranteeing the freshness and authenticity of the product to lowering the production costs. To this aim, a common "cheap practice" is the use of long refrigerated milk or "stored" curd in mixture with or as a replacement of the freshly prepared curd. The "stored" curd is a semi-finished product, with a low moisture content that, being stored under freezing conditions, allows shortening of the cheese

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production cycle (Faccia et al., 2014). These practices are not mandatory for the purpose of labelling, but they offer an economic gain, since the stored curds and long-refrigerated milk lower the costs related to the management of fresh milk.

On this topic, Faccia et al. (2014) studied the formation of the α_{S1}-casein fragment (f24–199), known as α_{S1}-I-CN, resulting from the chymosin activity on α_{S1}-CN (Carles & Ribadeau-Dumas, 1985; McSweeney, Olson, Fox, Healy, & Hojrup, 1993) and demonstrated that the quantification of this fragment by urea-alkaline electrophoresis can be used to reveal the use of stored curd in fiordilatte manufacturing. The authors recommended the α_{S1}-I-CN quantification to assess the production of the cheese from fresh milk under appropriate manufacturing conditions.

The freshness of mozzarella made from milk of the Mediterranean river buffalo (*Bubalus bubalis* L.) was studied by Petrella, Nava, Gagliardi, la Gatta, and Di Luccia (2015). The investigation was focused on plasmin activity on β-CN that gives rise to the formation of γ-CN fragments. By an electrophoretic approach, this study led the authors to tentatively propose an 'index of freshness' based on the relationship between β-CN and its γ-CN fragments: γ_1-CN (f29–209), γ_2-CN (f106–209), γ3-CN (f108–209) and γ_4-CN (f69–209). Preliminary results produced a calculated 'index' that showed a low value for low-quality mozzarella (made from poor quality milk or stored at the wrong temperature and time conditions). Also, Rutigliano et al. (2022), in a deeper electrophoretic study based on the detection of β-CN and its fragments in PDO buffalo mozzarella cheese samples, obtained a reliable index to estimate the freshness. The values of the index (Φ) well fitted with the freshness level of the mozzarella samples during storage, so the authors proposed the use of this index to guarantee the highest quality of buffalo mozzarella cheese branded "Made in Italy".

The present paper reports results of a study aimed at evaluating the level of 'freshness' of high moisture bovine mozzarella by assessing the proteolytic changes during storage. The study tested cheese samples manufactured under known conditions and stored at different temperatures for different times and unknown samples purchased from the market. The specific aim of the study was: (i) evaluating the relationship between β-CN and γ-CN fragments in known samples and (ii) evaluating the index of freshness (Φ) and the α_{S1}-I-CN formation in different conditions of storage; (iii) based on the obtained results, assessing the status of freshness of unknown samples.

2. Materials and methods

2.1. Samples

The HMMC samples considered for the investigation were divided into two main groups: the first group included samples used in the first stage of the study (named "Control Line") aiming at assessing the index of Freshness (Φ) and the formation of α_{S1}-I-CN (f24-199); these samples were manufactured in a dairy company located in Gioia del Colle (Bari, Italy) with the PDO certification "Mozzarella di Gioia del Colle".

The second group included samples purchased from dairy companies located in the province of Foggia (5 samples) and 53 samples of unknown origin delivered to our laboratory in three different periods (July, October and December 2020) by the Italian Finance Police; these samples were tested to prove the information obtained from the first stage of the study.

2.2. Experimental design and conditions of the experiment

The samples of the first group (PDO mozzarella) were stored for different times at room temperature (22.0 ± 1 °C), under refrigerated conditions (4 ± 2 °C) and frozen (−20 °C).

Sampling was done as follows: (i) samples stored at room temperature (RT) were analysed at the arrival to the laboratory (time 0) and after 1, 3, 5, 7, 14 days; (ii) samples stored at 4 °C, were analysed after 3, 5, 7, 14, 21 days of storage with (B) and without brine (WB); (iii) samples kept at −20 °C were analysed after 1, 3, 5, 7, 14, 21 and 28 days of storage without brine (WB). These samples were separated from the brine and transferred into sterilised polyethylene bags before freezing, because the brine should have exerted a negative impact on product properties (Alinovi & Mucchetti, 2020).

The thawing of the frozen samples was performed by keeping them in bowls at room temperature (22.0 ± 1 °C) for about 2 h, which was the time needed to cut them easily. All samples were analysed in triplicate and, before analysis, they were empirically checked for texture integrity by checking the level of softening through sight and touch and by evaluating cutting resistance; successively pH was measured with a pH-meter GLP 22 (Crison Instruments, Hach Lange S.L.U., Derio, Spain). The temperature was assessed with a Data Logger KT-220-O (Kimo Instruments, Castel San Pietro Terme, Bologna, Italy).

The samples of the second group were immediately sampled and analysed upon arrival to the laboratory.

2.3. Sodium dodecyl-sulphate-polyacrylamide gel electrophoresis

An aliquot of HMMC (0.1 g) was solubilised in 1.0 mL of 8 M urea and the protein content was determined using the 2D Quant-Kit™ from GE-Healthcare (GE-Healthcare Bio Sciences, Little Chalfont, UK) according to supplier's instructions. Determinations were done in duplicate and 20 μg of protein was taken for the electrophoretic analysis. They were diluted (1:1, v/v) with Laemmli buffer made of 0.0625 M Tris–HCl, pH 6.8, 2% SDS and 10% (v/v) glycerol. The analysis was performed under reducing conditions in the presence of 5% (w/v) dithiothreitol (DTT), using bovine purified casein, obtained according to the method of Di Luccia et al. (2009), as standard.

Electrophoresis was carried out on 12% polyacrylamide gels (8.6 × 6.7 cm) using a Mini-Protean tetra cell (Bio-Rad, Hercules, CA, USA). The gels were stained with 0.25% (w/v) Coomassie Brilliant Blue G-250 (CBB) overnight and de-stained with water. Prestained SDS-PAGE standards (Bio-Rad, Richmond, CA, USA) were used as protein molecular mass markers.

Image analysis of the gels was carried out using Quantity One 1-D software (Bio-Rad). The program was set to auto-scale all the gels on the same grey-scale and the trace quantity was used as parameter. This factor is defined by the integration of the signal intensity over the width and height of each band (Intensity × mm) and reflects the band intensity.

2.4. Alkaline Urea-PAGE

The above cheese suspension was mixed (1:1, v/v) with urea sample buffer (Tris–HCl pH 8.6, 8 M urea, traces of bromophenol blue sodium salt) and analysed under reducing conditions (2.5% DTT). Samples were loaded onto a 7.5% polyacrylamide gel (8.6 × 6.7 cm) using a Mini-Protean tetra cell (Bio-Rad) using the bovine purified casein, as standard. The analysis was conducted as reported by Andrews (1983), the gels were stained with CBB and de-stained in water.

Image analysis was carried out using Quantity One 1-D software (Bio-Rad). The program was set to auto-scale all the gels on the same grey-scale and the relative quantity (%) of the band related to α_{s1}-I-CN was used as parameter. This factor reflects the quantity of a band measured by its intensity, expressed as a percentage of the total intensity of all the bands in the lane.

2.5. Statistical analyses

Experimental data were compared by one-way analysis of variance (ANOVA). A Tukey's test with the option of homogeneous groups ($P < 0.05$), was carried out to determine significant differences between samples. Pearson correlation coefficients (r) and principal component analysis were considered to study relationships among variables. All statistical analyses were performed using XLSTAT 2020.1 software (Addinsoft, New York, NY, USA).

3. Results and discussion

3.1. SDS-PAGE of the 'Control line' samples and determination of the index of freshness (Φ)

The electrophoretic patterns of the samples tested at different temperature for different times of storage are shown in Fig. 1.

The electrophoretic profiles of RT samples (Fig. 1A) revealed the main bands related to the casein fractions: α_{S2} and α_{S1}-CN (estimated mass 23.6 kDa and 25.2 kDa, respectively), β-CN (estimated mass 23.9 kDa), γ_1-CN (estimated mass 20 kDa) and γ_2-CN (estimated mass 12 kDa) + γ_3-CN (estimated mass 11.5 kDa) and para-κ-CN (about 14 kDa). As expected, the profile after 14 days of storage was characterised by an intense proteolysis, with a clear decrease of β-CN band intensity (Fig. 1A) and the presence of several secondary bands originated from the parental casein fractions.

The electrophoretic profile of the samples stored at 4 °C without brine (WB, Fig. 1B) did not show noticeable differences over time, since from the day 1 of storage, all bands showed the presence of γ-CNs fragments (γ1-CN and γ2+3-CN). Basically, the same trend was observed for the samples stored in refrigerated conditions with brine (B, Fig. 1C). The same trend was observed in the mozzarella samples stored at −20 °C (Fig. 1D), except for the presence of a band between 20 and 15 kDa that appeared after 7 days of storage. In fresh cheeses, like mozzarella, primary proteolysis of α_{S1}-CN with formation of α_{S1}-CN fragments depends on the activity of chymosin and microbial proteases (Di Matteo, Chiovitti, & Addeo, 1982; Farkye, Kiely, Allshouse, & Kindstedt, 1991), whereas β-CN primary proteolysis mainly depends on plasmin activity with formation of γ-CNs fragments (Baruzzi, Lagonigro, Quintieri, Morea, & Caputo, 2012; Nielsen, 2002), with the rate of proteolysis influenced by the temperature.

A feature arising from all the electrophoretic profiles in all the storage conditions was the presence of a band, above that of para-κ-CN, with an estimated molecular mass of about 15 kDa, which was not identified (n.i.). It presented the same estimated molecular mass of γ_4-CN of buffalo milk (Di Luccia et al., 2009), which is absent in bovine milk.

To quantify the decrease of β-CN and/or the increase in the γ-CN fragments intensity, the image analysis was carried out and the index of freshness (Φ) was calculated according to Rutigliano et al. (2022), applying the following formula:

$$\Phi = \frac{\beta CN}{\beta CN + \gamma 1 + (\gamma 2 + \gamma 3)CN} \; x \; 100$$

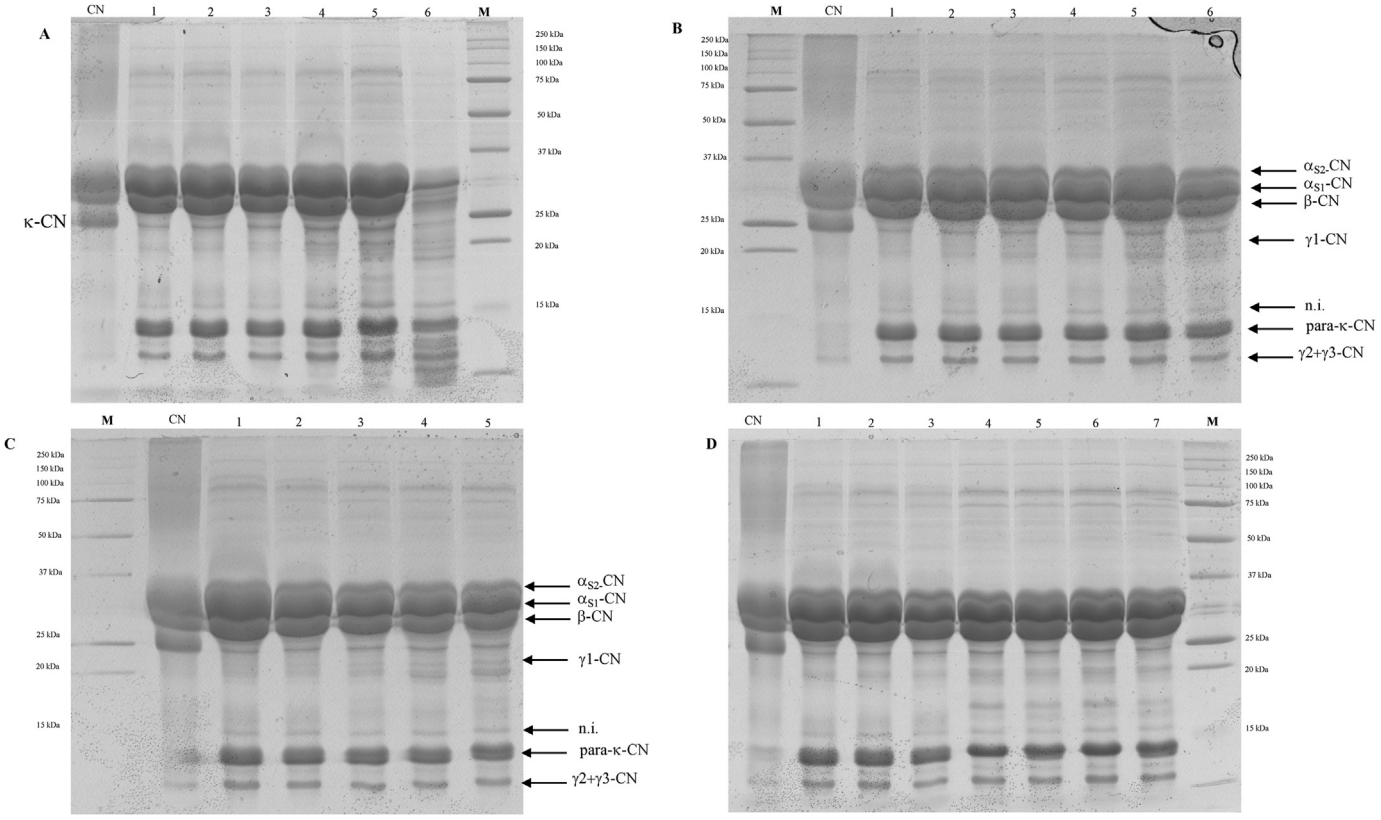

Fig. 1. SDS-PAGE of Control Line samples. Panel A, electrophoretic profiles of the samples stored at room temperature: CN, bovine casein; M, marker; lane 1, t0 sample; lane 2, t1 sample; lane 3, t3 sample; lane 4, t5 sample; lane 5, t7 sample; lane 6, t14 sample. Panel B, electrophoretic profiles of the samples stored in refrigerated (4 °C) conditions without brine: M, marker; CN, bovine casein; lane 1, t1 sample; lane 2, t3 sample; lane 3, t5 sample; lane 4, t7 sample; lane 5, t14 sample; lane 6, t21 sample. Panel C, electrophoretic profiles of the samples stored in refrigerated (4 °C) conditions with brine. M, marker; CN, bovine casein; lane 1, t3 sample; lane 2, t5 sample; lane 3, t7 sample; lane 4, t14 sample; lane 5, t21 sample. Panel D, electrophoretic profiles of the samples stored in freezing (−20 °C) conditions: M, marker; CN, bovine casein; lane 1, t1 sample; lane 2, t3 sample; lane 3, t5 sample; lane 4, t7 sample; lane 5, t14 sample; lane 6, t21 sample; lane 7, t28 sample.

where βCN was the intensity value of the β-CN band and $\gamma 1 + (\gamma 2 + \gamma 3)$CN were the intensity values of the γ-CN bands. The results of Φ were expressed as percentage.

A prospect of the rate of β-CN proteolysis for the PDO HMMC samples stored at different temperature, through the Φ assessment, is shown in Fig. 2.

A decreasing linear trend was observed for all the tested temperature of storage: room temperature (Fig. 2A, $R^2 = 0.92$), 4 °C without brine (WB) (Fig. 2B, $R^2 = 0.88$), 4 °C with brine (B) (Fig. 2C, $R^2 = 0.94$) and −20 °C (Fig. 2D, $R^2 = 0.86$).

The slope was used to understand the decreasing trend of the data (Φ) and to assess proteolysis. In this regard, being the assessment of the index of freshness strictly related to the formation of γ-CN fragments, the highest negative trend was registered for the samples stored at room temperature (slope: −4.20) from t0 to t7, whereas the slope was lower (−2.66) considering the interval from t0 to t14. Therefore, the percentage of γ-CN fragments formation increased of 53% from t0 to t7, while decreased (19.2%) considering the whole time of storage (from t0 to t14). This difference was due to the progression of proteolysis, in fact as shown in Fig. 1A, the bands related to γ_1-CN and $\gamma_2 + \gamma_3$-CN disappeared or had a lower intensity at t14, as a consequence of the formation of several secondary bands.

The samples stored with or without brine highlighted different decreasing trends, being more marked for the WB (slope: −9.19, after 1 day of storage; slope: −1.39, after 21 days of storage) than B samples (slope: −2.49 after 3 days of storage; slope: −1.20, after 21 days of storage). Moreover, in the case of refrigerated storage, the percentage of increase of the considered γ-CN fragments in WB was higher than B (53.5% versus 46.9%) considering the whole time of storage (from t0 to t21).

At −20 °C, the slope of the curve was more prominent (slope: −2.58) after three days of storage (t3) and then is constant until t28 (slope: −0.72). Additionally, at −20 °C, the percentage of γ-CN fragments formation increased of 41% considering the whole time of storage (from t0 to t28).

Summing up the main results of the assessment of Φ for the PDO samples it was found that.

(i) samples stored at room temperature (Fig. 2A) had a value that varied from 72.37% ± 5.69 (t0) to 35.16% ± 3.96 (t14), keeping a value above 60% until the third day of storage (59.82% ± 1.65). In this condition of storage, pH significantly changed only after 14 days, reaching the value 6.04 ± 0.1, probably in connection with microbial growth;

(ii) samples stored refrigerated without brine (WB) (Fig. 2B) had a value that remained above 60% until 3 days of storage (62.02 ± 2.47) and then decreased until 43.24 ± 5.3 after 21 days, with a concomitant increase of pH that reached the final value of 5.91 ± 0.09, because of a more intense proteolysis connected to a faster microbial activity. Differently, the value of the samples stored with brine (B) (Fig. 2C) remained higher than 60% for 7 days of storage (58.74 ± 2.32) and pH showed an increase from 5.56 ± 0.03 to 5.98 ± 0.01 after 14 days, time at which the cheese texture definitively deteriorated, passing from firm and elastic to very soft;

(iii) At −20 °C (Fig. 2D), values remained above the 60% until 14 days of storage (59.70 ± 1.09) and then decreased to 52.30 ± 1.47 after 28 days of storage, differently from pH that did not significantly vary along this period. Confirming that, the structure of the cheese remained unchanged after thawing.

3.2. ANOVA test of SDS-PAGE results

ANOVA test was performed separately on the quantity of the casein fractions analysed by SDS-PAGE (β-CN, γ_1-CN and γ_2+γ_3-CN)

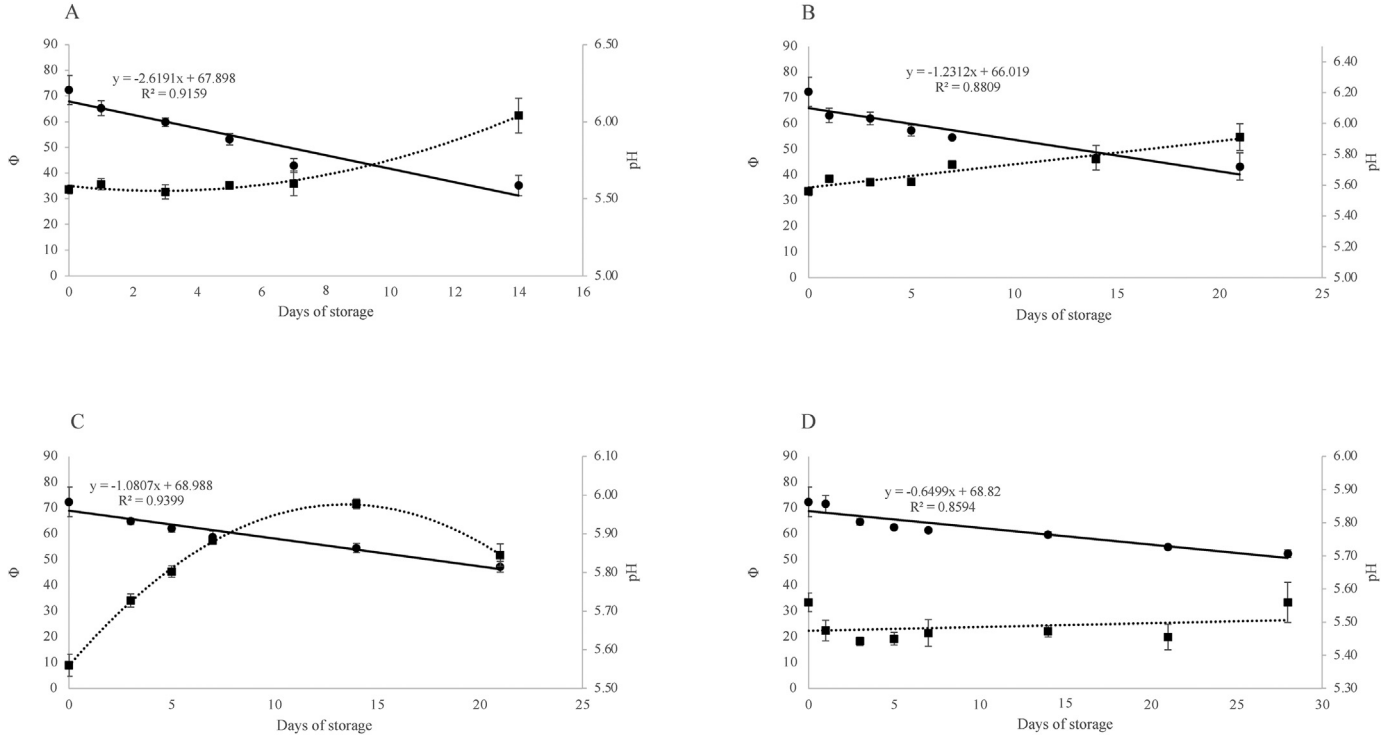

Fig. 2. Trends of pH (■) and index of freshness (Φ; ●) along the storage at (A) room temperature, (B) 4 °C without brine, (C) 4 °C with brine and (D) −20 °C. Solid lines represent the trend of the index of freshness; dashed lines represent the trend of pH along the storage.

and on the index of freshness (Φ) evaluated at different temperature (RT, 4 °C without and with brine, −20 °C) for different times of storage. The results are shown in Table 1.

At room temperature, β-CN did not show significant changes along the storage up to 7 days of storage (t7), whereas the value at t14 was statistically lower than the others ($P < 0.05$). As concern γ-CN fragments, γ_1-CN and $\gamma_{2+}\gamma_3$-CN values started to change significantly ($P < 0.05$) after 3 days and 5 days, respectively, showing further decrease at t14, when samples were characterised by a very intense proteolysis, which gave rise to smaller secondary fragments, probably from γ-CN (Fig. 1A and Table 1). In these conditions of storage, the index of freshness (Φ) discriminated samples after three days of storage (t3), becoming more effective after seven days (t7).

At 4 °C, β-CN values did not show significant differences, in agreement with the results of Gagliardi et al. (2009) and Rutigliano et al. (2018, 2022). In WB samples, the values of γ_1-CN significantly changed after 14 days (t14) of storage, while $\gamma_{2+}\gamma_3$-CN became statistically different ($P < 0.05$) after 5 days (t5). Samples stored with brine (B) showed the same tendency for γ_1-CN, while $\gamma_{2+}\gamma_3$-CN values became significantly different after 7 days (t7). In both these cases, the index of freshness (Φ) discriminated samples after 5 days of storage (t5).

Finally, for the samples stored at −20 °C, γ_1-CN started to change significantly already after 1 day, while $\gamma_2+\gamma_3$-CN were significantly different ($P < 0.05$) after 5 days. No significant differences emerged for β-CN. The index of freshness started to discriminate samples after 3 days of storage (t3).

The differences in the increasing rate of γ-CNs suggested that, according to the temperature of storage, the plasmin activity changed but microbial activity should be considered, too.

As matter of fact, β-CN fragments formation can be summed up as follows.

(i) at RT, γ_1-CN increased of 16.0% from 0 to 14 days and $\gamma_2+\gamma_3$-CN increased of 132.0% in the same period. This percentage can be explained taking into account the great reduction of β-CN band intensity after 14 days of storage, as shown in Fig. 1;
(ii) at 4 °C without brine, γ_1-CN increased of 33.3% from 0 to 21 days and $\gamma_2+\gamma_3$-CN increased of 60.2% in the same period;
(iii) at 4 °C with brine, γ_1-CN increased of 49.0% from 0 to 21 days and $\gamma_2+\gamma_3$-CN increased of 24.3% in the same period;
(iv) at −20 °C, γ_1-CN increased of 18.4% from 0 to 28 days and $\gamma_2+\gamma_3$-CN increased of 34.3% in the same period.

It could be inferred that at RT the proteolysis was directed towards γ_2 and γ_3-CNs formation, at 4 °C without brine (WB) there was a consistent formation for both γ_1-CN and for γ_2 and γ_3-CNs, but with these last with a percentage higher than the former. On the contrary, along the storage at 4 °C with brine (B) the fragmentation towards γ_1-CN formation was prominent, while at −20 °C, as expected, a slower fragmentation for both γ_1-CN and for γ_2 and γ_3-CNs was found.

Overall, the results obtained from the assessment of the index of freshness (Φ) implied that freshness was related to the microbial

Table 1
Trace quantity values of β-CN and γ-CNs bands measured with image analysis at different temperature for different days of storage and related index of freshness (Φ).[a]

Temperature (°C)	Storage (day)	β-CN	γ_1-CN	γ_{2+3}-CN	Φ
RT	0	7531.17 ± 235.79^{a}	384.88 ± 67.31^{d}	2518.43 ± 777.94^{c}	72.37 ± 5.69^{a}
	1	7388.10 ± 1336.32^{a}	470.38 ± 49.25^{c,d}	3420.14 ± 479.50^{c}	65.34 ± 2.90^{a,b}
	3	7006.39 ± 591.64^{a}	554.05 ± 28.41^{b,c}	4141.19 ± 127.29^{b,c}	59.82 ± 1.65^{b,c}
	5	7242.66 ± 277.09^{a}	634.20 ± 27.39^{b}	5760.39 ± 647.17^{b}	53.17 ± 2.27^{c}
	7	7285.78 ± 1346.82^{a}	1402.95 ± 50.26^{a}	8188.68 ± 758.39^{a}	42.96 ± 2.66^{d}
	14	2300.51 ± 62.53^{b}	486.70 ± 38.55^{c,d}	3804.34 ± 713.10^{c}	35.16 ± 3.96^{d}
	21	n.d	n.d	n.d	n.d
	28	n.d	n.d	n.d	n.d
4 °C (WB)	0	7531.17 ± 235.79^{a}	384.88 ± 67.31^{b}	2518.43 ± 777.94^{a}	72.37 ± 5.69^{a}
	1	7696.00 ± 633.32^{a}	384.30 ± 114.81^{b}	4187.07 ± 629.36^{a,b}	63.19 ± 2.82^{a,b}
	3	7827.06 ± 351.95^{a}	467.19 ± 228.15^{b}	4344.61 ± 490.44^{a,b}	62.02 ± 2.47^{ab}
	5	7210.99 ± 762.75^{a}	571.65 ± 235.23^{b}	5320.29 ± 421.22^{b,c}	57.24 ± 2.08^{b,c}
	7	7436.56 ± 856.76^{a}	580.34 ± 255.67^{b}	5659.63 ± 914.56^{b,c}	54.61 ± 0.30^{b,c}
	14	7624.10 ± 725.03^{a}	2530.51 ± 717.05^{a}	62745.08 ± 1445.70^{c,d}	46.48 ± 4.78^{c,d}
	21	7254.15 ± 695.58^{a}	2789.56 ± 739.91^{a}	6791.74 ± 1155.00^{d}	43.24 ± 5.30^{d}
	28	n.d	n.d	n.d	n.d
4 °C (B)	0	7531.17 ± 235.79^{a}	384.88 ± 67.31^{c}	2518.43 ± 777.94^{b}	72.37 ± 5.69^{a}
	1	n.d	n.d	n.d	n.d
	3	7282.01 ± 217.00^{a}	530.22 ± 61.59^{c}	3413.06 ± 260.01^{a,b}	64.91 ± 1.18^{a,b}
	5	7283.12 ± 224.72^{a}	875.81 ± 103.42^{c}	3565.96 ± 286.22^{a,b}	62.16 ± 1.48^{b,c}
	7	7123.41 ± 287.54^{a}	1394.34 ± 607.65^{b,c}	3634.72 ± 191.84^{a}	58.74 ± 2.32^{b,c}
	14	7330.11 ± 467.19^{a}	2353.86 ± 576.86^{b}	3795.72 ± 284.76^{a}	54.48 ± 1.80^{c,d}
	21	7187.49 ± 306.44^{a}	3887.57 ± 391.76^{a}	4148.25 ± 109.12^{a}	47.23 ± 2.10^{d}
	28	n.d	n.d	n.d	n.d
−20 °C	0	7531.17 ± 235.79^{a}	384.88 ± 67.31^{e}	2518.43 ± 777.94^{d}	72.37 ± 5.69^{a}
	1	7706.48 ± 221.44^{a}	693.65 ± 65.39^{d}	2382.30 ± 434.41^{d}	71.56 ± 3.31^{a,b}
	3	7442.50 ± 182.85^{a}	825.61 ± 76.30^{c,d}	3247.66 ± 164.02^{c,d}	64.64 ± 1.22^{b,c}
	5	7699.19 ± 279.86^{a}	977.87 ± 54.05^{b,c}	3642.77 ± 42.13^{c}	62.48 ± 0.94^{c}
	7	7845.14 ± 150.06^{a}	1001.68 ± 56.73^{b,c}	3940.01 ± 29.88^{b,c}	61.35 ± 0.53^{c,d}
	14	7753.84 ± 277.14^{a}	1197.58 ± 33.58^{b}	4034.50 ± 27.05^{b,c}	59.70 ± 1.09^{c,d}
	21	7522.49 ± 251.72^{a}	1636.75 ± 96.60^{a}	4561.16 ± 26.36^{a,b}	54.82 ± 0.84^{d,e}
	28	7611.64 ± 202.75^{a}	1790.79 ± 191.04^{a}	5152.48 ± 81.96^{a}	52.30 ± 1.47^{e}

[a] Abbreviations are: WB, without brine; B, with brine. Trace quantity expressed as Int × mm × 10^{-4} ± SD (n = 3). Significant differences ($P < 0.05$) are expressed with superscript letters within a column for each tested temperature; n.d, not detected.

quality of the raw matter used, the initial content of γ-CNs and by the time and temperature of storage used. These latter parameters, influencing the solubility of β-CN (Post, Arnold, Weiss, & Hinrichs, 2011) could affect the rate of proteolysis and the consequent formation of γ-CNs. Additionally, Alinovi, Wiking, Corredig, and Mucchetti (2020) found that the thawing phase of HMMC, stored under freezing conditions was a critical factor to control, because the modification of the casein structure influenced the rate of proteolysis favouring the enzymatic activity of plasmin and/or residual coagulating enzyme.

3.3. UREA-PAGE of the 'control line' samples and determination of α_{S1}-I-CN

Alkaline UREA-PAGE was performed to reveal the presence of α_{S1}-I-CN fragment (f24–199) in the samples and to evaluate the rate of formation in the different conditions of storage. Fig. 3 shows the electrophoretic patterns of samples stored at room temperature (Fig. 3A), at 4 °C without brine (WB, Fig. 3B) and with brine (B, Fig. 3C), and at −20 °C (Fig. 3D). The pattern showed that the intensity of the band related to α_{S1}-I-CN increased in terms of relative quantity percentage (%RQ) in all the storage conditions, but at different rate. The increase followed a linear trend (Fig. 4), but the slope of the curves depended on the storage conditions. The greatest increase was observed in the samples stored at room temperature, where %RQ varied from 0.98 ± 0.13 (t0) to 16.28 ± 0.31 (t14) (Table 2), (slope of the curve: 1.1). Actually, the %RQ value started to be significantly different with respect to zero time after 5 days.

At refrigerated conditions (4 °C), a similar trend during the whole time of storage was found for WB and B samples, and the slopes of the curves were quite similar (slope 0.43 and 0.45, respectively). Nevertheless, a remarkable increase in α_{S1}-I-CN formation was evident after 5 days in WB and after 14 days in B (Table 2).

At freezing conditions (−20 °C), as expected, the rate of formation of α_{S1}-I-CN was slower than the other conditions (slope of the curve: 0.1), reaching the final value of 4.32 ± 0.58 and showing more stable values throughout the storage. It is noteworthy that according to the results of Faccia et al. (2014), the highest α_{S1}-I-CN value for "fiordilatte" obtained from fresh curd was about 4RQ ("tolerance level").

To have a comprehensive view of the proteolytic phenomena, principal component analysis (PCA) was carried out to investigate

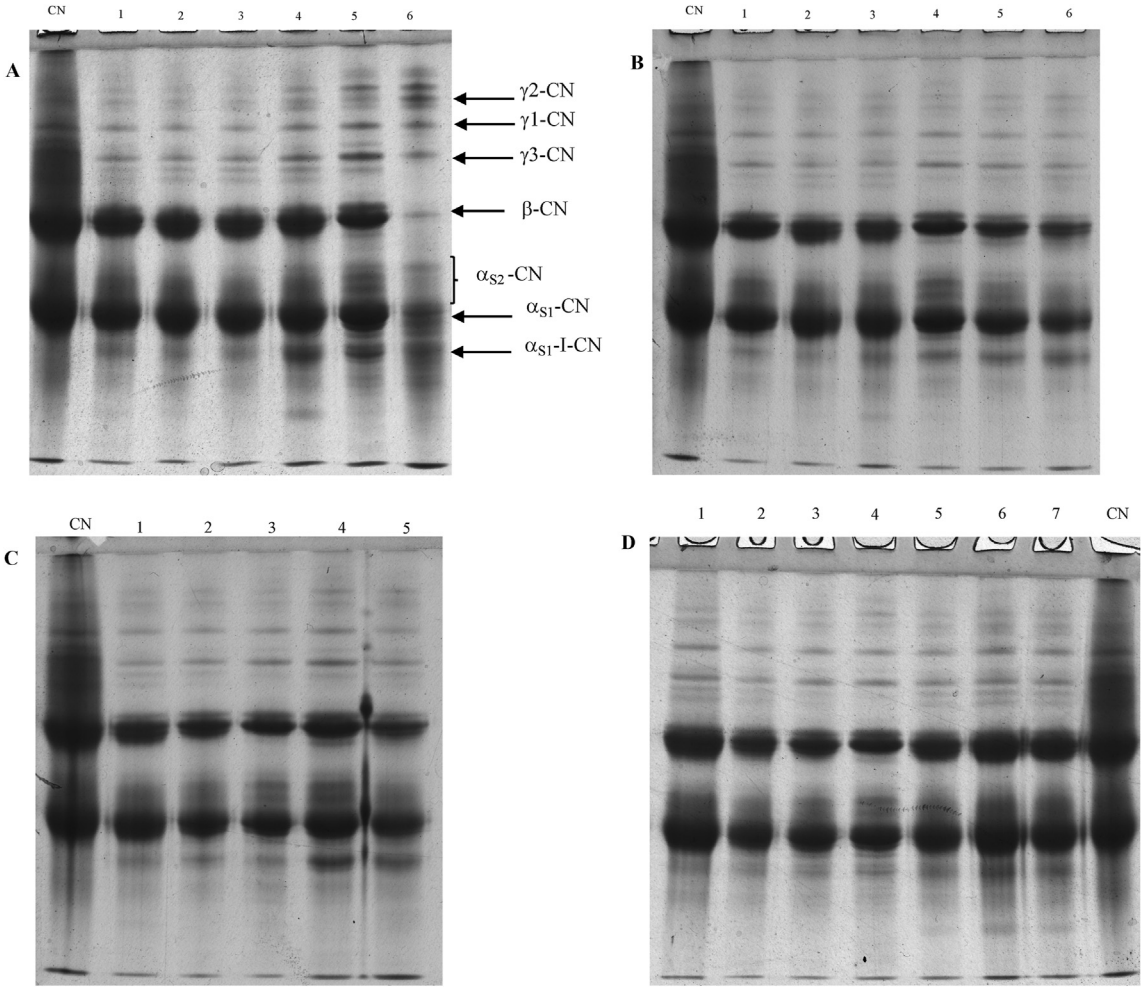

Fig. 3. Alkaline UREA-PAGE of Control line samples. Panel A, electrophoretic profiles of the samples stored at room temperature after 14 days of storage: CN, bovine casein; lane 1, t0 sample; lane 2, t1 sample; lane 3, t3 sample; lane 4, t5 sample; lane 5, t7 sample; lane 6, t14 sample. Panel B, electrophoretic profiles of the samples stored in refrigerated (4 °C) conditions without brine after 21 days of storage: CN, bovine casein; lane 1, t1 sample; lane 2, t3 sample; lane 3, t5 sample; lane 4, t7 sample; lane 5, t14 sample; lane 6, t21 sample. Panel C, electrophoretic profiles of the samples stored in refrigerated (4 °C) conditions with brine after 21 days of storage: CN, bovine casein; lane 1, t3 sample; lane 2, t5 sample; lane 3, t7 sample; lane 4, t14 sample; lane 5, t21 sample. Panel D, electrophoretic profiles of the samples stored in freezing (−20 °C) conditions after 28 days of storage: CN, bovine casein; lane 1, t1 sample; lane 2, t3 sample; lane 3, t5 sample; lane 4, t7 sample; lane 5, t14 sample; lane 6, t21 sample; lane 7, t28 sample.

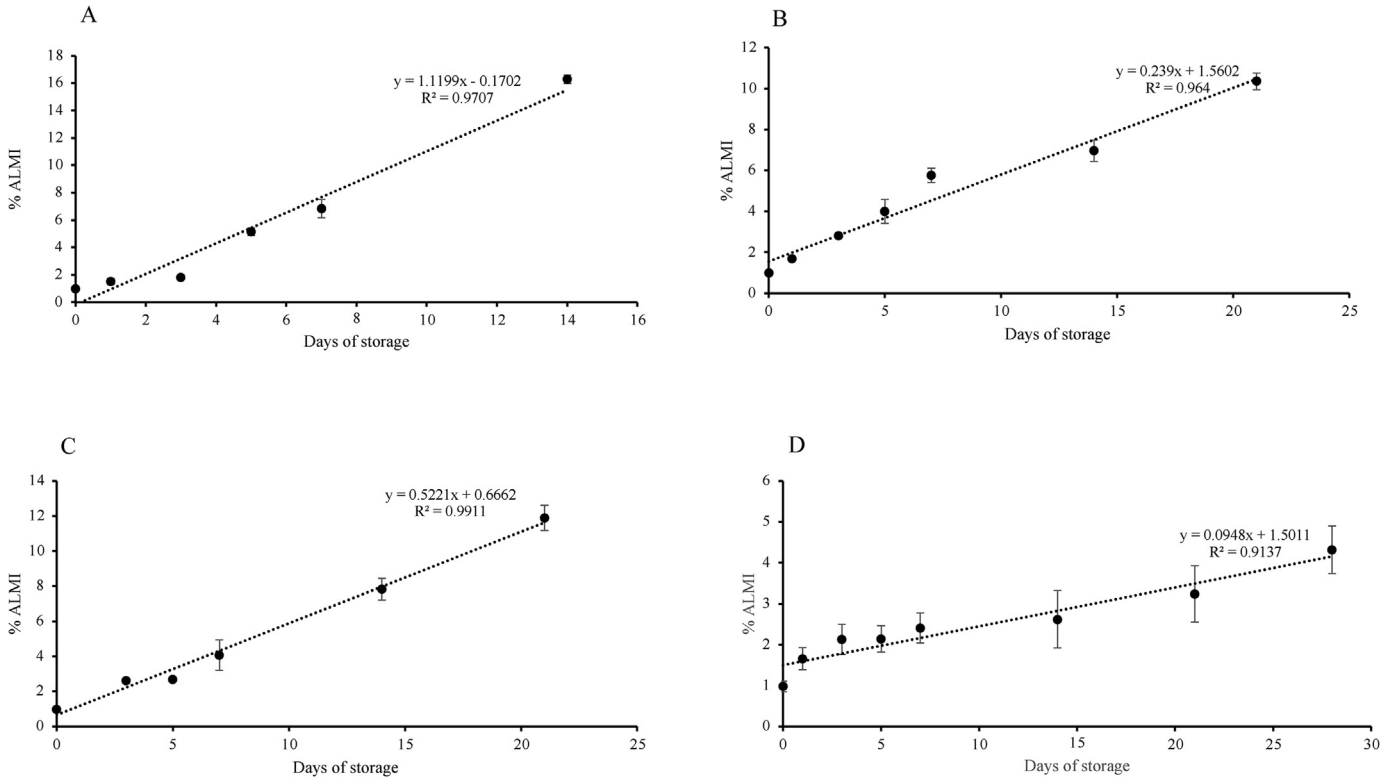

Fig. 4. Kinetic of formation of the α_{S1}-I-CN fragment during storage at (A) room temperature, (B) refrigerated (4 °C) conditions without brine, (C) refrigerated (4 °C) conditions with brine and (D) freezing conditions (−20 °C). Dashed lines represent the regression curve.

Table 2
Relative quantity (%) of the α_{S1}-I-CN band measured with image analysis at different temperature for different days of storage.[a]

Temperature (°C)	Storage (day)	α_{S1}-I-CN (f24−199)	Temperature (°C)	Storage (day)	α_{S1}-I-CN (f24−199)
RT	0	0.98 ± 0.13^{d}	4 °C (WB)	0	0.98 ± 0.13^{f}
	1	1.52 ± 0.21^{d}		1	1.69 ± 0.07^{f}
	3	1.81 ± 0.05^{d}		3	2.80 ± 0.09^{e}
	5	5.16 ± 0.29^{c}		5	4.00 ± 0.58^{d}
	7	6.83 ± 0.66^{b}		7	5.76 ± 0.35^{c}
	14	16.28 ± 0.31^{a}		14	6.96 ± 0.52^{b}
	21	n.d		21	10.35 ± 0.41^{a}
	28	n.d		28	n.d
4 °C (B)	0	0.98 ± 0.13^{e}	−20 °C	0	0.98 ± 0.13^{d}
	1	n.d		1	$1.66 \pm 0.27^{c,d}$
	3	2.63 ± 0.04^{d}		3	$2.13 \pm 0.37^{b,c,d}$
	5	$2.70 \pm 0.12^{c,d}$		5	$2.14 \pm 0.32^{b,c,d}$
	7	4.07 ± 0.87^{c}		7	$2.41 \pm 0.37^{b,c}$
	14	7.83 ± 0.62^{b}		14	$2.62 \pm 0.70^{b,c}$
	21	11.89 ± 0.72^{a}		21	$3.24 \pm 0.69^{a,b}$
	28	n.d		28	4.32 ± 0.58^{a}

[a] Abbreviations are: WB, without brine; B, with brine. Relative quantity is expressed as percentage ± SD (n = 3). Significant differences ($P < 0.05$) are expressed with superscript letters within a column for each tested temperature; n.d, not detected.

the correlations among the variables considered for the calculation of the index of freshness (β-CN, γ_1-CN and $\gamma_{2+}\gamma_3$-CN), α_{S1}-CN and α_{S1}-I-CN. PCA generated four graphs (Fig. 5) that explained the variance of the samples for each considered condition of storage. Samples were analysed in triplicate so the active variables in the score plot were three for each time of storage. The score plot of the samples stored at room temperature is shown in Fig. 5A. From the Pearson correlation matrix, it can be inferred that the highest and more significant positive correlation was found between γ_1-CN and $\gamma_{2+}\gamma_3$-CN (0.89), followed by β-CN and (0.83). Two factors were extracted explaining 55.02% and 39.19% of the variance, respectively, with a cumulated explained variance of 94.21%. From the score plot, it was evident the clear separation of the samples according to their time of storage. Fresh samples showed a negative correlation with both F1 and F2, while samples with higher time of storage (t ≥ 7days) were on the right part of the score plot, positively correlated with F1 and with γ_1-CN and $\gamma_{2+}\gamma_3$-CN. These results might indicate how F1 could be related to the evolution of proteolysis along the storage, with the progressive formation of γ-CN fragments, the disappearing of β-CN (Fig. 1A) at t14. As matter of fact, these variables were in a completely different quadrant, far from all the others.

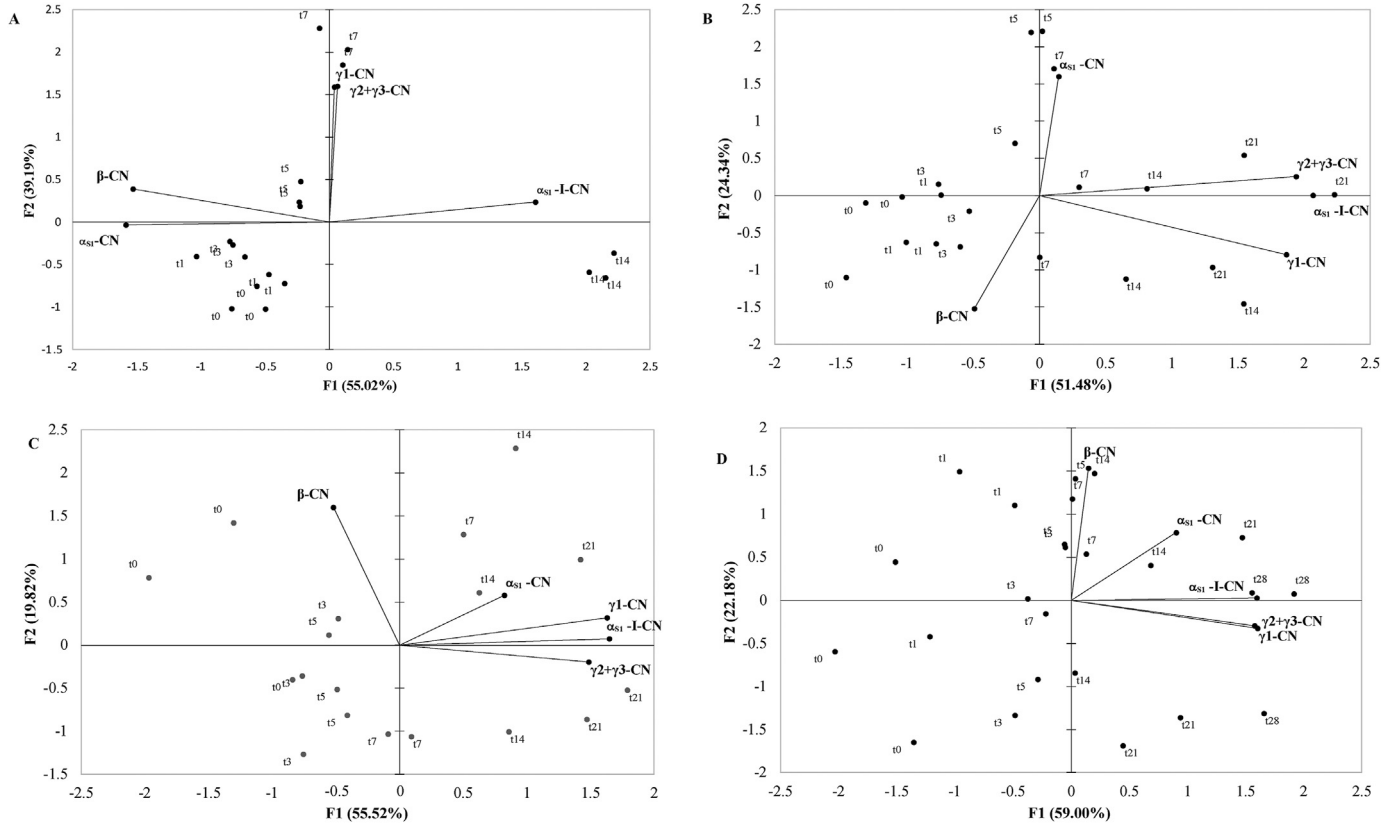

Fig. 5. PCA of the high moisture mozzarella cheese samples stored at (A) room temperature, (B) refrigerated (4 °C) conditions without brine, (C) refrigerated (4 °C) conditions with brine and (D) freezing (−20 °C) conditions.

At refrigerated conditions, without (Fig. 5B) and with (Fig. 5C) brine, the Pearson correlation matrix showed that a significant positive correlation was found between γ_1-CN and $\gamma_{2+}\gamma_3$-CN for both WB (0.66) and B (0.7) samples. A significant and positive correlation was found also between γ-CNs and α_{S1}-I-CN for both the refrigerated conditions of storage (Fig. 5B and C), higher for γ_1-CN than $\gamma_2+\gamma_3$-CN (0.83 and 0.95, respectively). The score plots of both WB and B samples showed two principal components, which explained 75.82% and 75.34% of the total variance, respectively. Also in these cases, a clear separation occurred according to the time of storage, with fresh samples (t < 5days) positively correlated with β-CN and negatively with F1. Samples with longer time of storage (t ≥ 7days) were positively correlated with F1 and with the γ-CN fragments and α_{S1}-I-CN, even if a more evident dispersion of the active variables was observed.

Finally, the Pearson correlation matrix of samples stored at −20 °C showed a significant correlation, higher than all the other tested temperature, between γ_1-CN and $\gamma_{2+}\gamma_3$-CN (0.9), followed by a positive correlation between γ-CNs and α_{S1}-I-CN, with a similar magnitude (≅ 0.8). The active variables were discriminated according to the time of storage (t ≥ 7days) and the two extracted factors (F1 and F2) explained 81.18% of the total variance. Also in this case, the fresh samples were negatively correlated with F1, contrary to samples with t < 7days and all the active variables with t > 14days were around the loadings of the γ-CNs and α_{S1}-I-CN (Fig. 5D).

Therefore, as general feature, we could individuate the factor F1 as indicator of proteolytic activity, which means indigenous and microbial enzyme actions. Moreover, the F2 component could individuate the production of fragments derived both from the enzymatic action on parent protein and on their fragments.

If we compare the results of fiordilatte obtained from this experimentation and PDO Mediterranean buffalo mozzarella cheese, according to the results of Rutigliano et al. (2022), the index of freshness for PDO buffalo mozzarella cheese was 70% after 1 day of storage at RT, as in the case of fiordilatte. PDO buffalo mozzarella cheese showed Φ >70% at 4 °C and −20 °C still after 3 days and 7 days, respectively, differently, from the values obtained for fiordilatte, which instead showed much lower values (Φ <65%) already after 1 day of storage. These differences can be explained considering the structural architecture of the products and the organisation of the protein network, determined by changes in the primary structure, percentage of casein fractions and a higher protein content that allow to bound much more water, being this last proportional to the protein content of the cheese (McMahon, Fife, & Oberg, 1999).

3.4. Assessment of the index of freshness and of α_{S1}-I-CN (f24–199) in unknown samples

The information obtained with 'Control line' experimentation was applied to the unknown samples purchased from the market and/or from local dairy industries. The results obtained from the image analysis of the electrophoretic patterns of SDS-PAGE and alkaline urea-PAGE (gels not shown) were used to calculate the index of freshness and to assess the relative quantity percentage (RQ%) of α_{S1}-I-CN. As it can be seen from Table 3, most of the samples (65.5%) had an index of freshness (Φ) higher than 65% and

Table 3
Index of freshness (Φ) and α_{S1}-I-CN fragment levels of the unknown samples.[a]

Sample	Φ (%)	α_{S1}-I-CN (% RQ)	Sample	Φ (%)	α_{S1}-I-CN (% RQ)
1	65.72	2.15	30	65.09	2.97
2	64.18	2.49	31	64.18	3.74
3	60.25	2.95	32	68.25	3.70
4	62.9	3.70	33	70.66	2.98
5	61.44	2.41	34	69.31	6.18
6	58.91	6.42	35	63.69	3.91
7	61.44	2.65	36	68.17	3.79
8	63.84	3.04	37	71.08	4.40
9	62.45	6.25	38	68.16	2.16
10	65.71	1.99	39	69.40	2.02
11	65.37	4.00	40	69.89	2.56
12	59.04	6.28	41	70.22	2.29
13	66.63	3.00	42	68.53	2.28
14	55.74	6.95	43	68.96	2.19
15	63.05	1.97	44	71.57	2.36
16	66.63	3.87	45	72.60	2.49
17	64.46	4.43	46	73.46	2.46
18	63.99	5.28	47	71.64	2.59
19	68.31	3.17	48	72.06	2.67
20	69.48	3.01	49	71.83	2.77
21	63.33	2.84	50	75.50	3.01
22	67.55	3.58	51	72.40	3.73
23	67.92	3.29	52	73.37	2.74
24	65.60	3.29	53	73.82	3.05
25	62.71	2.98	54	62.19	4.88
26	67.25	3.39	55	61.89	2.40
27	68.46	3.13	56	61.46	2.64
28	65.54	3.83	57	74.12	3.74
29	66.90	2.76	58	72.74	3.58

[a] Abbreviation: % RQ, relative quantity expressed as percentage.

%RQ related to α_{S1}-I-CN lower than 4%. Only 5% of the samples showed an index of freshness (Φ) lower than 60% and %RQ > 5% and among them, three samples (n. 6, 12 and 14), showed both a lower index of freshness and a higher %RQ related to α_{S1}-I-CN, indicating poor conditions of the raw matter and/or improper conditions of storage. Sample 34 can be highlighted because showed both a high value of Φ and a high value of α_{S1}-I-CN. These values may indicate the use of stored curd with a low moisture content for the manufacturing of mozzarella cheese.

4. Conclusion

The present work was focused on the application of the electrophoretic method to evaluate a reliable index for estimating the freshness of bovine mozzarella. Based on the results obtained by evaluating the γ-CN and α_{S1}-I-CN fragments formation in known samples, trends were obtained describing the variation of the index of freshness and α_{S1}-I-CN under controlled conditions, which could help to correctly evaluate the status of the product. A value higher than 60% is maintained up to 3 days at RT, 5 days at 4 °C, with and without brine and about 14 days at −20 °C. Comparing these results with those of the unknown samples taken from the market, it can be observed that the data of the index of freshness and α_{S1}-I-CN were consistent. Most of the samples showed at the same time a high index of freshness (Φ) and a low %RQ related to α_{S1}-I-CN, allowing confirmation of the 'freshness' of the samples.

Further investigation will aim to assess the characteristics of those samples that can show both a high index of freshness and % RQ related to α_{S1}-I-CN, to evaluate the possible use of stored curd or semi-finished products.

Declaration of competing interest

None.

Data availability

Data will be made available on request.

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