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18 **Abstract**

19
20 Freshness is one of the more appreciated attributes for high moisture Buffalo mozzarella cheese that
21 guarantees the highest quality for the consumer. However, the freshness decreases during the life of
22 this cheese due to proteolysis events caused mainly by milk endogenous enzymes, such as plasmin.
23 Plasmin generated the γ -CNs fragments from the β -CN proteolysis that can be assessed by SDS-
24 PAGE technique and the image analysis. The β -CN decrease and γ -CNs increase represent, therefore,
25 useful markers to obtain an index of freshness (Φ). For this purpose, a “Control Line” was assessed
26 analyzing Buffalo Mozzarella Cheese (BMC) with the PDO certification. The samples were stored
27 under different conditions (room temperature, refrigerated and frozen) for different times. Moreover,
28 145 BMC samples purchased from markets were studied to understand if the calculated Φ was
29 consistent with the results of the Control Line study. The obtained results demonstrated that the BMC
30 samples positively correlated with the Φ had the highest freshness level, whereas the samples
31 correlated to γ -CNs had the lowest one. This work provided a reliable method to evaluate the
32 freshness conditions of PDO buffalo mozzarella cheese assessing the proteolysis mechanism
33 concerning the formation of γ -CNs fragments during the storage of this Italian typical product.

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35

36 **Introduction**

37
38 High-Moisture mozzarella (HMM) is a typical fresh cheese of Southern Italy obtained from Italian
39 Mediterranean Buffalo (IMB) or bovine milk. Historically, the cheese was developed in Campania
40 region to recover spontaneously acidified IMB milk (Alinari, 1915). The processing technology has
41 basically unchanged overtime: after milk coagulation, the curd is acidified by natural starters, minced
42 and added with hot water to be “stretched” and molded by hand. From this latter operation, which is
43 called in Italian “*mozzare*” (*to chop off*), derives the name “*mozzarella*”. The sensory features of
44 buffalo mozzarella are spherical shape, white porcelain colour, smooth, bright and humid surface,
45 and pleasant, slightly sour flavour (Gianferri et al., 2007; Aponte et al., 2010). On 12 June 1996, the
46 cheese obtained the Protected Denomination Origin (PDO) trademark as “Mozzarella di Bufala
47 Campana” according to Commission Regulation (EC) no. 1107/1996. According to the official
48 production protocol, Buffalo Mozzarella Cheese (BMC) must be produced from raw or pasteurized
49 milk within 60 hours after milking. In the last decades, the PDO-labelled product has spread
50 worldwide and is now appreciated almost everywhere, giving rise to a production volume worth over
51 1,2 billion euros (Svimez, 2019). It is best consumed as a fresh cheese and, for this reason, it is stored
52 in a covering liquid in order to preserve freshness, avoid the formation of a rind and maintain the
53 typical flavour. Due to the high moisture content, BMC presents a short shelf-life; the expected
54 sensory attributes by consumers are cohesivity, acidity and saltiness (Pagliarini et al., 1997;
55 Mucchetti, Pugliese, & Paciulli, 2016). The evaluation of freshness of BMC has become very
56 important to assure the best quality to consumers. To this purpose, Petrella et al. (2015b) tentatively
57 proposed a ‘freshness index’ throughout an electrophoretic study that allowed to establish a
58 relationship between β -CN and its γ -CN fragments to discriminate fresh BMC from non-fresh one.
59 In particular, the lowest the value of the index, the worst the quality of the raw matter/mozzarella
60 and/or the longest the time of storage. Those preliminary results allowed to consider the calculated
61 ‘index’ as a tool to assess how proteolysis could be related to the freshness of the cheese. On the other
62 hand, several studies showed that the formation of casein fragments and their content may indicate

63 whether fresh cheeses, such as buffalo mozzarella, have been produced and/or stored according to
64 the good standards of processing technology (Farkye et al. 1991; Di Luccia et al. 2009; Faccia et al.
65 2014; Petrella et al., 2015b).

66 It is known that the extent of casein degradation in cheese is closely related to the activity of
67 endogenous proteases (mainly plasmin and cathepsin) and of residual rennet enzymes (chymosin and
68 pepsin) (Mc Sweeney et al., 1995; Kelly et al. 2006). Plasmin degrades all caseins, but it is active
69 especially on β -CN (Eigel et al., 1984; Fox et al., 1994) and α_{s2} -CN (Le Bars and Gripon, 1989). The
70 primary cleavage sites for this enzyme in β -CN are Lys28-Lys29, with the formation of the fragment
71 f29–209 (γ 1-CN) with a molecular weight of 20521 Da; Lys105-His106, with the formation of the
72 fragment f106–209 (γ 2-CN) with a molecular weight of 11822 Da; and Lys107-Glu108, with the
73 formation of the fragment f108–209 (γ 3-CN) with a molecular weight of 11556 Da (Somma et al.
74 2008). Moreover, buffalo milk is characterized by the presence of an additional γ -CN, due to a residue
75 of Lys at position 68 replacing Asn, present in the bovine counterpart. This amino acid substitution
76 generates the fragment 69-209, the so called γ 4-CN, with a molecular weight of 15748 Da (Trieu-
77 Cuot & Addeo, 1981; Di Luccia et al., 2009).

78 Chymosin was found to be active both on α_{s1} -CN and β -CN in solution (Carles & Ribadeau-Dumas,
79 1984), but mainly it hydrolyzes α_{s1} -CN in cheese (McSweeney et al., 1993; Mulvihill and McCarthy,
80 1993; Sienkiewicz et al., 1994). The primary site of cleavage for chymosin on α_{s1} -CN is the bond
81 Phe23-Phe24, with the formation of the fragment f24–199, known as α_{s1} -I-CN (Carles & Ribadeau-
82 Dumas, 1985; McSweeney et al., 1993) and this action was reported that could be responsible for the
83 softening of the cheese (De Jong, 1976; Creamer and Olson, 1982). The quantification of the fragment
84 α_{s1} -I-CN, evaluated by Faccia et al., (2014), was found to be useful in the assessment of the use of
85 stored curd for high-moisture bovine mozzarella cheese production.

86 In order to contribute to the evaluation of the freshness of PDO Buffalo Mozzarella cheese and to
87 check the storage time as a characteristic inferable from the production and expiry dates showed on
88 the label, a study was undertaken on the formation of casein fragments in BMC samples stored at

89 different times and temperature (room, refrigerated and freezing conditions). Therefore, the study
90 aimed at evaluating the relationship among β -CN, the casein proteolysis products (γ -CN fragments)
91 and the calculated index of freshness (Φ).

92

93 **2. Materials and Methods**

94 *2.1 Experimental design*

95 The experimental design considered two different phases. The first one, called “Control Line”, was
96 based on the analysis of nine batches of BMC that were purchased from dairy companies adhering to
97 the production specification established by the Consortium for the Protection of Mozzarella di Bufala
98 Campana PDO cheese. From each batch, six samples ($48.16\text{g} \pm 1.19$ in weight each) were kept at
99 room temperature (RT, $22.0 \pm 1^\circ\text{C}$), seven ($46.67\text{g} \pm 3.26$) were kept refrigerated at 4°C and seven
100 ($47.12\text{g} \pm 1.79$) were frozen (-20°C).

101 The batches were sampled for analyses during storage as follows:

- 102 - immediately (time 0) and after 1, 3, 5, 7 and 14 days (at room temperature);
- 103 - after 1, 3, 5, 7, 14, 21 and 28 days of storage (kept refrigerated);
- 104 - after 1, 3, 5, 7, 14, 21 and 28 days of storage (kept frozen).

105 All samples were purchased directly from the dairy companies in the early morning within a period
106 of one month. The measurement of the temperature of the samples was done by Data Logger KT-
107 220-O (Kimo Instruments, Castel San Pietro Terme, Bologna).

108 The samples at room temperature were stored in their original bags, immersed in their brine, until
109 analysis, while the refrigerated and frozen samples were separated from the covering liquid and
110 transferred in sterile polyethylene bags for storage.

111 As regards the frozen samples, thawing was performed by keeping them in bowls at room temperature
112 ($22.0 \pm 1^\circ\text{C}$) until it was possible to observe a smooth surface, easy to cut (about 2h). All BMC
113 samples used in this phase of the experimentation were sampled and analyzed in triplicate.

114 Before analysis, the pH value of all samples was measured with a pH-meter GLP 22 (Crison
115 Instruments, Hach Lange S.L.U., Derio, Spain).

116 The second phase of the experimentation consisted in testing the information obtained from the
117 Control Line phase on a significant number (145) of unknown samples of BMC purchased from
118 supermarkets within a period of about two years (beginning of 2017- mid 2019). The samples were
119 divided into three batches, according to the purchasing time: batch 1 (2017, 52 samples); batch 2
120 (2018, 74 samples); batch 3 (2019, 19 samples). Immediately after the purchase, the samples were
121 sampled and kept at -20°C until analysis.

122

123 *2.3 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS PAGE) and Alkaline Urea* 124 *Polyacrylamide Gel Electrophoresis (Urea PAGE).*

125 Each BMC sample (0.1 g) was solubilized in 1.0 mL of 8 M Urea and the protein content was
126 determined using the 2D Quant-Kit™ from GE-Healthcare (GE-Healthcare Bio Sciences, Little
127 Chalfont, UK) according to supplier's instructions. Each quantification was done in duplicate and 30
128 µg of proteins were loaded onto the gel.

129 For SDS-PAGE, the protein extracts were diluted (1:1 v/v) with Laemmli buffer (0.0625 M Tris–
130 HCl, pH 6.8, 2% SDS, 10% glycerol). All samples were analyzed under reducing conditions
131 (presence of 5% dithiothreitol, DTT) using a 15% polyacrylamide (8,6 x 6,7 cm) gel.

132 For Alkaline Urea-PAGE, the protein extracts were mixed with 100 µL of Urea sample buffer (Tris-
133 HCl pH 8.6, Urea 8M added with a few drops of bromophenol blue sodium salt solution) and analyzed
134 under reducing conditions (2.5% DTT) using a 4 - 7.5 % polyacrylamide gel (8,6 x 6,7 cm). As
135 reference, a bovine and a buffalo purified caseins, obtained according to Di Luccia et al., (2009) were
136 loaded onto the gels that were run in a Mini-PROTEAN tetra cell (Bio-Rad, Hercules, CA).

137 The gels were stained with 0.25% w/v Coomassie Brilliant Blue G-250 (CBB) overnight and de-
138 stained with water. Prestained SDS-PAGE standards (Bio-Rad, Richmond, CA, USA) was used as
139 protein molecular weight markers.

140

141 *2.4 Image analysis of the SDS-PAGE patterns*

142 Image analysis was carried out using Quantity One 1-D software (Bio-Rad, Hercules, CA). The
143 program was set to auto-scale all the gels on the same grey-scale and the trace quantity was used as
144 parameter. This parameter defined the quantity of a band as measured by the area under its intensity
145 profile curve. Units are intensity x mm.

146

147 *2.5 Statistical analyses*

148 To evaluate the effects of the different times and storage conditions (room temperature, refrigeration
149 and freezing), data were statistically processed by ANOVA test. Principal component analysis (PCA)
150 was performed on the variables (β -CN, γ 4-CN, γ 1-CN γ 2+ γ 3-CN and the calculated index of
151 freshness). Pearson correlation coefficients (r) were calculated to find relations among the evaluated
152 variables. All the statistical analyses were performed using XLSTAT 2020.1 software (Addinsoft,
153 New York, N.Y., USA).

154

155 **3. Results**

156 *3.1 Electrophoresis analyses of the samples of the Control Line phase.*

157 The evolution of proteolysis in the samples was first studied through the assessment of the increasing
158 formation of γ -CN fragments overtime by SDS PAGE. No marked differences emerged among the
159 samples of the nine batches, so the electrophoretic profiles of one representative sample are shown in
160 Figure 1 A, B and C. The bands related to the proteolysis products were already observed in the fresh
161 samples (Figure 1A, lane 2). The most intense band was that of γ 4-CN (estimated MW 15 kDa),
162 whereas γ 1-CN (estimated MW 20 kDa) and γ 2-CN (estimated MW 12 kDa) + γ 3-CN (estimated
163 MW 11.5 kDa) occurred as faint bands, becoming more evident after three days of storage. As
164 expected, the electrophoretic profiles of the samples analyzed after 14 days of storage at room

165 temperature were characterized by an intense proteolysis, showing more evident bands of all γ -CN
166 fragments.

167 The electrophoretic profile of the samples stored at 4°C for 1, 3, 5, 7, 14, 21 and 28 days are shown
168 in Figure 1B. The profiles did not show noticeable differences among each other, since they all
169 showed the presence of γ -CNs fragments (γ 1-CN, γ 4-CN and γ 2+ γ 3-CN) from day 1 and the variation
170 of the intensity of the bands seemed comparable during the storage. Basically, the same trend was
171 also observed for the samples stored at freezing conditions (Figure 1C).

172 By using Alkaline Urea-PAGE technique, it was possible to observe the proteolysis of α _{s1}-CN
173 fraction and, in particular, the formation of the α _{s1}-I-CN fragment (f25-199). It is known that the
174 formation of this proteolytic product is mainly caused by the activity of residual rennet (Faccia et al.,
175 2014). The detection of the α _{s1}-I -CN fragment was clearly evident from day 7 onwards at RT,
176 whereas at 4°C it was barely visible only on day 28 and completely absent at -20°C. The
177 electrophoretic profiles of seven representative samples are shown in Figure 2 A, B and C.

178

179 3.2 Determination of an index for assessing BMC freshness

180 The decrease of β -CN and /or the increase in the γ -CN fragments intensity is not fully appreciable
181 just observing the electrophoretic profiles, therefore the image analysis was carried out. The
182 proportion of γ -CNs and β -CN bands were obtained evaluating the parameter 'Trace Quantity' for
183 each band of each electrophoretic profile, obtained by three different electrophoretic analysis. The
184 obtained values were used to calculate the index of freshness (Φ) and plotted vs the days of storage.
185 For this purpose, we considered that the whole β -CN fraction was composed of the sum of γ -CN
186 fragments and the remaining parental β -CN, thus the Φ index was expressed as %, through the
187 formula:

188

189
$$\Phi = \frac{\beta\text{CN}}{\beta\text{CN} + \gamma 1 + \gamma 2 + \gamma 3 + \gamma 4} \times 100$$

190

191 Considering the slope of the three linear curves, a perspective of the proteolysis rate of the samples
192 can be drawn. Actually, a decreasing linear trend was observed for the BMC samples stored at room
193 temperature (Figure 3A; $R^2 = 0,97$), at 4°C (Figure 3B; $R^2 = 0,88$) and at -20 °C (Figure 3C; $R^2 =$
194 0,90), with the highest negative slope for the data obtained at RT (slope: -1,50, from t0 to t14). Under
195 refrigerated condition, the slope of the straight line was the highest after the first day of storage (slope:
196 -3,63) and then the decreasing trend was quite constant from day 3 (-1,54) until day 21 (-0,44),
197 reaching a steady state between day 21 and day 28 (-0,4). Also, the slope of the straight line under
198 freezing condition was the highest after the first day of storage (slope: -2,0) and then the decreasing
199 trend was quite constant from day 3 (-0,7) until day 21 (-0,3), reaching a steady state between day 21
200 and day 28 (-0,27).

201 Considering the whole time of storage for the three different temperatures (14 days at room
202 temperature and 28 days at refrigerated and freezing conditions), the difference of the percentages
203 between the slopes at RT and 4 °C was 73,7%, between RT and -20°C was 81,7%, and between 4°C
204 and -20 °C was 30,3%.

205 As expected, the proteolysis rate was higher at RT than at 4°C and -20°C, as demonstrated by the
206 remarkable difference of the percentages, while a lower difference was found between 4°C and -
207 20°C.

208 The main results obtained from the 'Control line' samples experimentation can be gathered as follow:

209 - Room Temperature: the freshness of the BMC samples, *in sensu stricto*, can be considered
210 from 75,11%±2,30 to 69,83%±3,04, a trend that occurred in three days of storage. After this
211 period, the freshness decreased with a concomitant increase of pH ($\text{pH } 5.40 \pm 0.04$) up to the
212 53,59%±1,25 with the loss of the structure ($\text{pH } 7,05 \pm 0,09$) (Fig. 3A) after 14 days. In this
213 regard, from a structural point of view, BMC samples stored at room temperature in their
214 brine, showed the softening of the body after 7 days of storage and a collapse after 14 days.

- At refrigerated conditions (4°C): the freshness of the BMC samples, *in sensu stricto*, can be considered up to five days of storage (from 75,11%±2,30 up to 69,75%±0,41) (Figure 3B).
- At -20°C, the freshness could be considered prolonged until 14 days (from 75,11%±2,30 up to 68,84%±1,94) (Figure 3C).

In the last two cases, the pH was enough constant with an average of $5.6 \pm 0,10$ in the former and about $5.4 \pm 0,04$ in the latter. The occurrence of the slightest variation of pH did not influence the structure of the cheeses, albeit the index of freshness (Φ) marked a proteolysis in progress, but to a less extent than that at RT (Figure 3A, B and C).

3.3 Statistical analysis

3.3.1 Anova TEST

ANOVA test was carried out in order to assess the differences among the samples stored at different temperature (room temperature, 4°C and -20°C) for different times, using as variables the trace quantity of the γ -CNs ($\gamma 1$ -CN, $\gamma 4$ -CN and $\gamma 2+\gamma 3$ -CN) and β -CN bands and the index of freshness (Φ). The results of ANOVA test are reported in Table 1. At room temperature, no significant differences were found in the trace quantity values of β -CN throughout storage (from t0 to t14). On the other hand, Gagliardi et al., (2009) and Rutigliano et al., (2018) demonstrated that the detection of the actual decrease of β -CN during a considered time of storage of mozzarella cheese was feasible only through the ELISA technique. Differently, differences were found for $\gamma 1$ -CN, $\gamma 4$ -CN and $\gamma 2+\gamma 3$ -CN fragments by electrophoretic analyses. As a matter of fact, the trace quantity of $\gamma 1$ -CN became statistically different already after 1 day of storage from t0, then the value was quite constant until the 14th day of storage, when it reached the highest value. As regards $\gamma 4$ -CN and $\gamma 2+\gamma 3$ -CN fragments, the trace quantities were found to be significantly different after 5 days of storage, reaching the highest values after 14 days of storage.

239 As a general feature, the index of freshness (Φ) did not allow to discriminate the fresh samples (t_0)
240 from the samples stored for 1 day (t_1) at RT, but it did after three days of storage (t_3). However, it
241 started to be effective after 5 days of storage in the sample discrimination and it was much more
242 effective after 7 days.

243 At 4°C, the ANOVA test showed no significant differences in the measured trace quantity of β -CN
244 throughout the considered time of storage (from t_0 to t_{28}). The trace quantities of γ_1 -CN and $\gamma_2+\gamma_3$ -
245 CN fragments, instead, were significantly different after 1 day and then, after a certain stability, they
246 became significantly different in the following 14 days of storage; while in the case of γ_4 -CN, a
247 significant difference was revealed after the first day of storage and then the values were no longer
248 statistically different. The index of freshness (Φ) allowed to significantly discriminate the samples
249 after 1 days of storage and it was more effective after 7 days of storage.

250 At freezing conditions (-20°C), the ANOVA test revealed no significant differences in the β -CN trace
251 quantity values along the storage with respect to the initial time (t_0). As concerns γ -CNs fragments,
252 γ_1 -CN and γ_2+3 -CN became significantly different after 7 days, while γ_4 -CN after 14 days. All the
253 trace quantities values of the γ -CNs fragments were significantly different from the initial value (t_0)
254 after 28 days of storage.

255 An important feature arose from the analysis of the index of freshness (Φ) which, on the contrary,
256 started to discriminate the buffalo mozzarella samples after 5 days of storage from the initial time
257 This difference became even more marked after 28 days of storage.

258

259 3.3.2 Assessment of proteolysis by PCA

260 Multivariate analysis was carried out for better understanding the correlations between β -CN, its γ -
261 fragments and the calculated Index of Freshness (Φ) in relation to temperature and time of storage.
262 The samples from the nine batches of “Control line” experimentation were divided into four groups
263 (the first three samples and then three groups made of two samples) and the averages of the obtained

264 data for each considered parameter (β -CN, its γ -fragments and the calculated Index of Freshness)
265 analyzed in triplicate was considered. Consequently, the active variables in the score plot are four for
266 each time of storage.

267 PCA generated three PCs (Figure 4A, B and C) that explained the variance of the dataset according
268 to the different times of storage for each considered temperature (room, refrigerated and frozen) and,
269 in all of the cases, the two principal components (F1 and F2) gathered most of the information
270 (94,76% Fig. A; 92,51% Fig. B; 85,56% Fig. C). In all graphs, F1 was strongly associated with the
271 values of the index of freshness (Φ), while β -CN was the dominant variable for F2. The high
272 correlation and the wide variability of the four considered variables (β -CN and γ -CN) didn't always
273 allow a clear discrimination of BMC samples.

274 The PCA of the samples stored at room temperature is shown in Figure 4A. From the Pearson
275 correlation matrix, it was inferred that the highest positive correlation is that between γ 4-CN with γ 1-
276 CN ($r=0,875$) and with γ 2+3-CNs ($r=0,872$), and between γ 1-CN with γ 2+3-CNs ($r=0,7$). As
277 expected, all γ -fragments showed an evident negative correlation with the index of freshness (Φ),
278 especially γ 1-CN ($r=-0,92$) and γ 4-CN ($r=-0,9$). Two factors were extracted, explaining 71,27% and
279 23,49% of the information, respectively, with a cumulated explained variance of 94,76%.

280 A positive correlation between F1 and the considered variables γ 4-CN, γ 2+ γ 3-CN, γ 1-CN and β -CN
281 was shown, while the principal component F2 showed a positive correlation with γ 4-CN, γ 2+3-CNs,
282 β -CN and Φ and a negative correlation with γ 1-CN, which was on the opposite sector of the plot with
283 respect to the Φ .

284 These data indicate that the proteolysis at RT was pushed towards the formation of the γ -fragments,
285 which negatively influence the index of freshness.

286 Therefore, the first principal component (F1), which contains most of the information, showed a
287 certain separation of the samples, based on the time of storage, explaining the extent of γ -CNs
288 formation. In this respect, considering the possible further fragmentation of γ 1-CN and γ 4-CN into

289 $\gamma_2+\gamma_3$ -CN, as well as the possible fragmentation of γ_1 -CN to γ_4 -CN, it might be hypothesized that β -
290 CN was preferentially hydrolyzed to $\gamma_2+\gamma_3$ -CN and γ_4 -CN and to a less extent to γ_1 -CN. Moreover,
291 a further fragmentation of γ_1 -CN primarily to the γ_4 -CN, which in turn was fragmented to $\gamma_2+\gamma_3$ -CN
292 can be hypothesized. It must be noted that the samples at t0 and t1 were on the opposite sector with
293 respect to γ -CNs, while the samples at the end of storage (t14) were concentrated around γ_1 -CN.
294 In this case, the proteolysis evolution indicated that samples nearer γ -CNs, hence more correlated to
295 them, were more proteolyzed.
296 From the experimentation carried out at 4°C (Figure 4B), it was noteworthy a specular score plot with
297 respect to RT. Actually, the Pearson correlation matrix showed that the highest positive correlation
298 was underlined between γ_4 -CN with $\gamma_2+\gamma_3$ -CN ($r=0,73$) and between γ_1 -CN with $\gamma_2+\gamma_3$ -CN ($r=0,68$).
299 A lower correlation was found between γ_4 -CN and γ_1 -CN ($r=0,55$) and β -CN with γ_4 -CN ($r=0,4$) and
300 $\gamma_2+\gamma_3$ -CN ($r=0,47$).
301 Also in this case, the negative correlation between the index of freshness and the γ -fragments was
302 confirmed, especially with γ_1 -CN ($r=-0,92$) that was also the only γ -fragment negatively correlated
303 with β -CN ($r=-0,4$).
304 With regard to the eigenvalues, the F1 component explained 62,36% of the information and the
305 second one (F2) 30,15%, describing 92,51% of the total variance. The score plot (Figure 4B) showed
306 a positive correlation between the two extracted factors (F1 and F2) and β -CN and the index of
307 freshness (Φ), while a negative correlation was found between F2 and γ_1 -CN.
308 At 4°C, it appeared that proteolysis mainly acted on β -CN and produced γ_1 -CN. Concomitantly, it
309 acted on γ_4 -CN to produce the smaller $\gamma_2+\gamma_3$ -CN fragments, considering the not significant
310 correlation of these fragments with β -CN ($r=0,151$). Therefore, a more intense proteolysis on the γ -
311 CN fragments rather than on β -CN was found at 4°C. Moreover, it must be noted that the samples at
312 t0 was opposed to γ -CNs, while the samples at the end of storage (t28) were concentrated around γ_1 -
313 CN.

314 From the experimentation carried out at -20°C (Figure 4C), the Pearson correlation matrix showed
315 that the highest positive correlation was underlined between the variables γ 4-CN and γ 1-CN ($r=0,67$),
316 followed by that with γ 2+ γ 3-CN ($r=0,61$). β -CN showed a negative correlation with all the γ -CNs
317 fragments, but actually significant only with γ 2+ γ 3-CN ($r=-0,51$). The index of freshness showed a
318 positive correlation with β -CN ($r=0,66$) and negative with all γ -fragments, with the same magnitude
319 ($r=-0,8$).

320 As regard the two extracted factors, the F1 component explained the 70,38% of the information and
321 F2 the 15,18%, with a cumulated explained variance of 85,56%. The score plot (Fig. 4C) showed a
322 positive correlation between F1 with β -CN and the index of freshness, which was on the same plane
323 of the factor ($r=0,999$). Only γ 2+ γ 3-CN showed a negative correlation with F2. The active
324 observations were clearly divided in the two parts of the score plot, if compared with F1: samples
325 with short times of storage were on the right (t0, t1, t3, t5, t7, positively correlated with F1) and
326 samples with long times of storage were on the left (t14, t21, t28, negatively correlated with F1). In
327 the former case we can hypothesize a possible proteolysis of β -CN to γ 4-CN and γ 1-CN, while the
328 second one suggested a slow hydrolysis of these fragments to γ 2+ γ 3-CN.

329 From these results, it seems that the actual decrease of the freshness and the evolution of proteolysis,
330 in these conditions of storage relied mainly on γ 4-CN and γ 1-CN, then on β -CN, which were slowly
331 fragmented to γ 2+ γ 3-CN.

332 In particular, a common trend was highlighted at all the analysed conditions of storage: longer times
333 of storage insisted on γ -CNs fragments variables. Therefore, it could be supposed that the γ -CNs
334 fragments variables are those that have the greatest weight to describe the samples with pronounced
335 proteolysis.

336 However, all the transformations were not discrete for the each considered time and temperature of
337 storage, thus we could observe, in some cases, a dispersion of the active observations.

338

339 3.4 Verification of the index of freshness on BMC samples found on the market by SDS-PAGE

340 In order to test the information obtained by the ‘control line’ study, 145 samples (divided into three
341 batches of analysis) purchased from supermarkets were analyzed through SDS-PAGE, to calculate
342 the index of freshness (Φ). All samples showed all the bands related to the γ -CN fragments (data not
343 showed) and the image analysis allowed to highlight the differences among them through the Φ
344 assessment (Table 2).

345 Multivariate analysis of these samples (Figure 5) showed that the variables were all positively
346 correlated among each other, especially β -CN with γ 4-CN ($r=0,912$) and with γ 2+ γ 3-CN ($r=0,846$).
347 The F1 component explained the 66,73% of the information and the component F2 the 23,38%, with
348 a cumulated explained variance of 90,11%. All variables were positively correlated with F1, whereas
349 only β -CN and the index of freshness were positively correlated with F2. Most of the samples of
350 batch 2 and batch 3 were grouped in the same zone of the plot, where it can be argued a slow
351 proteolysis, mainly due to enzymatic action focused on β -CN and γ 4-CN to produce γ 2+ γ 3-CN, and
352 to a less extent γ 1-CN. Differently, the samples of batch 1 were more separated from each other,
353 probably because of a higher variability in the proteolysis evolution.

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355 4. Discussion

356 Complying with the rules established by the production regulations for the PDO brand of Buffalo
357 Mozzarella cheese is the most important target to produce a high-quality product, universally
358 recognized and, consequently, protected at national and international level. One of the challenges for
359 high moisture cheeses is to establish the quality of raw matter, in terms of composition and microbial
360 load, and the freshness of the final product. The cheeses that present a high moisture content,
361 considering the short time for consumption are mostly subjected to primary proteolysis by the milk
362 endogenous proteases, mainly plasmin, which has a marked activity on β -CN, and by exogenous
363 enzyme, mainly chymosin from residual rennet acting primarily on α _{s1}-CN. For this purpose, some

works focused their attention on plasmin, with the detection of β -CN and γ -CN fragments proportions, and residual chymosin activity with the measurement of α_{s1} -I-CN fragment (Faccia et al.2014; Petrella et al., 2015a). In the case of buffalo milk, the formation of a fourth γ -CN fragment derived from β -CN (γ 4-CN, (f69-209) was individuated as the first proteolytic event, differently from the bovine counterpart, where the first event was the formation of γ 1-CN (Di Luccia et al., 2009). In a recent work, Rutigliano et al., (2018) underlined that the monitoring of the small degradation of the parental buffalo β -CN in refrigerated and frozen milk at different times was possible through an immunochemical method. Also, the application of mass spectrometry made possible to identify specific protein markers (β -Lactoglobulin, α -Lactalbumin, γ 2-, γ 3- and γ 4-caseins, β -casein-derived phosphopeptides (1–68) and GLYCAM1 phosphorylated peptides (f1–53)) related to the freezing storage of buffalo milk (Arena et al., 2016).

Alinovi et al. (2020~~x~~) studied, through electrophoretic and chromatographic techniques, the effects of frozen storage on high-moisture mozzarella cheese, highlighting a higher rate of proteolysis in the thawing phase following the frozen storage, as a consequence of enzymatic activity, enhanced by the casein supramolecular structures modifications.

A preliminary electrophoretic study (Petrella et al., 2015b) monitored the rate of formation of the γ -CN fragments, through the application of SDS-PAGE to evaluate a possible approach to assess the freshness of buffalo mozzarella cheeses at different temperature of storage. These authors proposed some ranges for the index of freshness, achieving a classification of the samples on the basis of their conservation state. The present study verified, through the assessment of the “Control Line” curves and statistical analysis, the validity of the proposed index of freshness and allowed to understand in detail the evolution of proteolysis through the monitoring of γ -CNs fragments, at different temperature of storage, since the β -CN decrease is not effectively detectable with the SDS-PAGE electrophoretic technique, as confirmed by our results (Table 1). Moreover, the results confirmed the detection of β -CN proteolysis in small extent with the electrophoretic techniques and the occurrence

389 of hydrolysis, mainly on γ -CN fragments. In this regard, interesting changes in the γ -CN formations
390 according to time and temperature of storage were found. Within this context, it is worth underlying
391 that γ 1-CN might give rise to γ 4-, γ 2- and γ 3-CN, having three “plasmin sensitive” sites: 68Lys-
392 69Ser, 106Lys-107His and 108Lys-109Asp, respectively, whereas γ 4-CN has only two sites 106Lys-
393 107His and 108Lys-109Asp producing γ 2- and γ 3-CN, respectively (Somma et al., 2008).

394 In addition to this, the UREA-PAGE analysis evidenced a further proteolysis on the α_{s1} -CN fraction,
395 probably, due to residual rennet activity, through the detection of α_{s1} -I-CN fragment that was clearly
396 evident at RT, barely visible at 4°C and completely absent at -20°C (Figure 2A, B, C), highlighting
397 a different behavior of this type of product with respect of its bovine counterpart, as showed by
398 Alinovi et al., 2020x.

399 As concerns the room temperature, it was clear that the rate of γ -CN formation was higher than in the
400 other cases and, on the basis of pH and the calculated Φ , we considered three days as limit of
401 freshness, *in sensu stricto*. As matter of fact, it was possible to calculate from the image analysis, how
402 there was an increase (29%) of γ -CNs at room temperature after 7 days of storage and 44% after 14
403 days. Differently, at refrigerated and freezing conditions, the rate of γ -CNs increase was quite lower
404 from t_0 to t_{28} , with 28% and 17% values, respectively.

405 Observing the curves of the “Control line” samples (Fig. 3A, B, C), the differences in the decrease of
406 the index of freshness (Φ) could be explained by referring them to the decrease of pH, the calcium
407 equilibrium and plasmin activity. At room temperature, a more consistent decrease of the pH values
408 was observed, which influenced the calcium equilibrium, where the insoluble colloidal form tends to
409 move towards the soluble ionic Ca^{2+} form. In this way, a change in the cheese structure was evident,
410 as already described by Alinovi et al., 2020x. This change in the structure of the product made γ -CN
411 fragments available to the action of residual plasmin, which is trapped in the calcium para-caseinate
412 network. This phenomenon could originate the different formation of γ 2- and γ 3-CN, deriving from
413 γ 1-CN and from γ 4-CN, which was assumed from our results.

414 We didn't observe the same rate of this phenomenon under refrigerated and freezing conditions, since
415 cooling slowed down both the shift of colloidal calcium towards Ca^{2+} and the plasmin activity, being
416 the decrease of pH less substantial, and the structure of the product unchanged.

417 As to the 145 samples bought from the market, the values of the index of freshness ranged from a
418 maximum of 79,59% (sample 65, batch 2) to a minimum of 50,7% (sample 4, batch 2). Overall,
419 34,5% of the samples had an index of freshness (Φ) >70,00% and 65,5% had an index of freshness
420 (Φ) <70,00%, but from these latest, only 21 samples (15%) had an index of freshness <60%. From
421 these data, it was inferred that most of the samples had an index of freshness that fit with the curves
422 obtained in the "Control line" experimentation, and only in few cases, lower or even higher values
423 than those calculated were registered. These results allowed to establish that most of the samples were
424 characterized by a lower formation of γ -fragments and a soft proteolysis of β -CN. For one of the
425 analyzed batches (batch 2), there was a wide variability of the index of freshness (Φ), with some
426 values much lower than 70%, because this batch was the largest one and, consequently, had probably
427 more variations in time and conditions of storage.

428 Based on this experimentation, considering that the index of freshness depends, basically, on the
429 variables that more strongly influence proteolysis in this type of cheese, i.e. raw matter quality and
430 storage conditions, BMC was considered to be fresh when Φ index was not lower than 70%.

431

432 **Conclusion**

433 The present work was focused on the assessment of an electrophoretic method to obtain a reliable
434 index for estimating the freshness of buffalo mozzarella cheese samples. On the basis of the obtained
435 results by evaluating the γ -CN fragments formation in the 'Control Line', trends were obtained that
436 described the variation of the index of freshness under controlled conditions of temperature and time
437 of storage, which can help to correctly evaluate the state of freshness of the product. A value higher
438 than 70% is maintained up to 3 days at RT, 5 days at 4°C and about 10 days at -20° C. Comparing

439 the results of the PCA of the samples from the market with those of the “Control Line” samples, it
440 can be observed that the position of vectors was similar to that of the RT and that the samples from
441 market near to the index of freshness (Φ) were those fresher than those farther that showed lower
442 values of the Φ . Finally, the values of the index of freshness (Φ) can well represents the mozzarella
443 cheese status and could be useful to indicate the consumer the highest quality of the Buffalo
444 Mozzarella cheese, to protect even more this PDO product, a flagship of the *Made in Italy* brand.

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